JOURNAL OF AGRICULTURAL RESEARCH

VOL. 78 WASHINGTON, D. C., JUNE 1, 1949 No. 11

THE ORIGIN OF BREEDS OF CHICKENS [1]

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INTRODUCTION

In Edward Brown's [2] monumental work entitled "Poultry Breeding and Production," he quotes an article by W. B. Tegetmeier, which appeared in the Field of September 26, 1885. In this article, Tegetmeier, who was an associate of Charles Darwin, questions Darwin's theory that all breeds of poultry have their origin in the wild jungle fowl, *Gallus gallus* (*G. furrugineus*). Brown (p. 5) quotes Tegetmeier as follows:

> But it is with regard to the Eastern Asiatic type of fowl . . . that my doubts as to the descent from the *G. furrugineus* are strongest. We have in the Cochin a fowl so different from the ordinary domestic birds that when first introduced the most ridiculous legends were current respecting it. Putting these on one side, we have a bird with many structural peculiarities that could hardly have been induced by domestication. Thus, the long axis of the occipital foramen in the Cochin is perpendicular, in our old breeds horizontal, a difference that could never have been bred for, and which it is difficult to see could be correlative with any other change. The same may be said respecting the deep sulcus or groove up the center of the frontal bone. The extraordinary diminution in the size of the flight feathers and that of the pectoral muscles could hardly have been the result of human selection and careful breeding, as the value of the birds as articles of food is considerably lessened by the absence of flesh on the breast. Nor is the extreme abundance of fluffy, soft body feathers a character likely to be desired in a fowl. The vastly increased size may have been a matter of selection, although, as the inhabitants of Shanghai feed their poultry but scantily, and, according to Mr. Fortune, mainly on paddy or unhusked rice, it is not easy to see how the size of the breed was obtained if, as is generally surmised, it arose from the little jungle fowl.
>
> Taking all these facts into consideration, I am induced to believe that the birds of the Cochin type did not descend from the same species as our game fowl. It may be asked what I would suggest as to the origin of these Eastern Asiatic breeds. In reply I would suggest the possibility, or even probability, of their being descended from some easily captured and really domesticated short-winged species that may have entirely passed into a state of domestication. . . .

These statements, though frequently quoted, have remained unchallenged and unsupported by any critical evidence for a considerable period of years. The senior author of the present report started several years ago to make a collection of skulls of different breeds and varieties of poultry with the object of checking upon the statements of Tegetmeier regarding the structural differences in the skulls. The focusing of attention in poultry breeding on relatively few breeds has resulted in some of the breeds in this collection becoming rare so that this series of skulls would be difficult to duplicate at the present time.

[1] Received for publication May 4, 1948. Contribution No. 175, Department of Poultry Husbandry, Kansas Agricultural Experiment Station.

[2] BROWN, E. POULTRY BREEDING AND PRODUCTION. 2 v., illus. London. 1929.

The collection contains a total of 245 skulls, representing 27 breeds and varieties of chickens. All skulls are from birds near or beyond the age of sexual maturity. Measurements were made of the vertical and horizontal axes of the foramen magnum (occipital foramen), and observations were made of the nature of the suture between the two frontals.

STRUCTURE OF THE FRONTALS

From Tegetmeier's statements it would appear that he had observed that the Asiatic chickens had a much more prominent groove between

TABLE 1.—*Data on structure of frontal bones and foramen magnum in 27 varieties of chickens*

Variety	Sex	Condition of sulcus between frontals			Mean ratio of axes of the foramen magnum
		Grooved	Flat	Ridged	
American class:					
Jersey Black Giant	Male		2		1.15
	Female	1			1.11
New Hampshire	do	2	3		1.15
Single-Comb Rhode Island Red	Male	10	4	1	1.15
	Female	26	14		1.15
Barred Plymouth Rock	Male	2	1		1.21
	Female	3	1		1.18
White Plymouth Rock	Male		1		1.15
	Female	2	2		1.16
White Wyandotte	Male	4			1.39
	Female	1	3	2	1.47
Silver Laced Wyandotte	do	1			1.15
	Unknown	5	3		1.15
Asiatic class:					
Light Brahma	Male	1	2		1.31
	Female	4			1.12
	Unknown	1			1.15
Buff Cochin	Female	3			1.05
Black Cochin	do	1			1.29
	Unknown	1			1.09
Partridge Cochin	Female	1			1.20
White Cochin	do	4			1.10
English class:					
Australorp	Male	1			1.35
	Female	7			1.29
Dark Cornish	Male	1			1.38
	Female	3			1.17
	Unknown	2			1.25
Buff Orpington	Male	1	1		1.34
	Female	2			1.34
White Orpington	Male		1		1.11
	Female	1			.82
Mediterranean Class:					
Ancona	do	2	1		1.17
Blue Andalusian	Male	2			1.30
	Female	2			1.12
Single-Comb Brown Leghorn	do	1	9	1	1.18
Single-Comb Buff Leghorn	Male			2	1.15
	Female	6	4		1.19
Single-Comb White Leghorn	Male	1	1	3	1.22
	Female	6	11	3	1.13
Single-Comb Black Minorca	Male		1	3	1.22
	Female	2	6		1.16
Single-Comb Buff Minorca	Male		1	3	1.32
	Female			1	.96
Single-Comb White Minorca	Male			1	1.55
	Female	5			1.15
	Unknown	1			.88
Miscellaneous:					
Breda	Male		1		1.13
Round Head game	Female	1			1.32
Indian jungle fowl	do		1		1.10
Barred Plymouth Rock-Rhode Island Red crossbred	do	14	4		1.13
White Leghorn-Rhode Island Red crossbred	do	5	8		1.19

the frontal bones of the skull than that found in other breeds of his day. It should be kept in mind, however, that several of the present-day breeds of the American and English classes were not known at the time he made his statements. Among the skulls examined in the present study were several of the Asiatic class, namely, the Light Brahma and the Buff, Black, Partridge, and White Cochins. Table 1 gives a list of the breeds and varieties studied, together with the quotient of the horizontal axis divided by the vertical axis of the foramen magnum. Mean ratios exceeding 1 indicate situations in which the horizontal axis is the longer and mean ratios of less than 1 indicate situations in which the vertical axis is the longer.

TABLE 2.—*Differences in classes of poultry in the structure of the frontal bones of the skull*

Breed group	Number of skulls	Percentage of frontals which are—				
		Extremely grooved	Moderately grooved	Slightly grooved	Flat	Ridged
American	94	3	17	40	36	3
Asiatic	18	28	39	22	11	----
English	20	5	30	55	10	----
Mediterranean	82	4	11	18	46	21

Table 2 shows the differences between four breed groups in the structure of the frontal bones of the skull. The data in table 2 show that the frontal bones may have grooves of varying depths, may be completely flat in that area, or may show a conspicuous ridge. (See fig. 1.) Since the numbers in each class differed widely, the distribu-

FIGURE 1.—Three skulls showing a flat, a deeply grooved, and a ridged suture between the paired frontal bones.

tion of the different types of frontals is expressed in percentage to facilitate comparisons, although it is recognized that some of the percentages are based on small numbers. The Asiatic groups not only showed a higher incidence of grooves between the frontals, but also possessed deeper grooves than the other groups.

The Mediterranean class showed less grooving between the frontals and a much higher incidence of ridges in the same area. Among the four breed and eight varietal representatives of the Mediterranean class, the Single-Comb White Leghorns and the Buff Minorcas showed the ridged frontals most conspicuously. A majority of the birds in the Mediterranean group failed to show any grooving of the frontals, which were either flat or ridged. The Asiatics had relatively few ungrooved frontals and were without ridges.

The representatives of the English class used in this study varied more in the direction of the Asiatics in lacking ridges and possessing grooved frontals. The grooving of the frontals of this group was less extreme than that of the Asiatics. The Dark Cornish had somewhat deeper grooves than the Australorps and Orpingtons.

The breeds and varieties of the American class were somewhat intermediate between the Asiatics and Mediterraneans with respect to the incidence of grooves and ridges. Of the seven varieties represented, the White Wyandottes varied most in the direction of the Asiatics.

In the miscellaneous group were females from the F_1 generation of crosses between the Barred Plymouth Rocks and Single-Comb Rhode Island Reds and also between Single-Comb White Leghorns and Single-Comb Rhode Island Reds. The pure Barred Plymouth Rocks and Single-Comb Rhode Island Reds did not differ much with regard to the frontal sulcus, and the crossbreds were similar to the parents. The Single-Comb Rhode Island Reds and the Single-Comb White Leghorns differed considerably in respect to the structure of the frontals, the Reds having only flat or grooved frontals with a majority grooved, while the Leghorns had more flat than grooved frontals and also some with ridges. The F_1 generation females from this cross showed only flat or grooved frontals wtih a majority of the latter type. No ridges appeared on the crossbreds, but the distribution of flats and grooves were more like that of the Leghorn, indicating dominance of the Leghorn type.

Table 3 presents an analysis of the relation of sex to the structure of the frontals. The representation of the two sexes was not equal, but the four classes of chickens had a fair number of each sex. In order to facilitate comparison, the distribution of the different types of frontals

TABLE 3.—*Relation of sex to skull structure*

Breed group	Number of skulls	Sex	Percentage of frontals which are—		
			Grooved	Flat	Ridged
American	25	Male	64	32	4
	61	Female	59	38	3
Asiatic	3	Male	33	67	
	13	Female	100		
English	5	Male	60	40	
	13	Female	100		
Mediterranean	19	Male	60	40	
	62	Female	100		

is expressed in terms of percentage. In all except the breeds in the American class, the females were exclusively in the group with grooved frontals. The males of all four classes had a considerable number with flat ungrooved frontals. This sexual dimorphism may have had some influence on the results shown in table 2, but in all classes of chickens represented, the number of female skulls greatly exceeded the males.

STRUCTURE OF THE FORAMEN MAGNUM

The vertical and horizontal axes of the foramen magnum were measured in all birds. This foramen, by which the spinal cord enters the skull, is somewhat variable in shape and dimensions. Its location and shape are shown in the posterior view of the skull given in figure 2. Owing to the sloping edges of the foramen, it is difficult to obtain exact measurements. In general, the practice was followed of taking the measurement at a point where it would give the maximum distance in the horizontal and vertical planes. The relative lengths of the axes were expressed by quotients of the horizontal axis divided by the vertical axis. Tegetmeier stated that the Asiatic chicken had its long axis in the vertical direction, and if expressed in the above-described manner, should have a value less than 1. Of the 245 individuals measured, only 9 had the longer axis of the foramen magnum in the vertical direction, and none of these was from the Asiatic class. A calculation of the mean ratios by classes shows the following results:

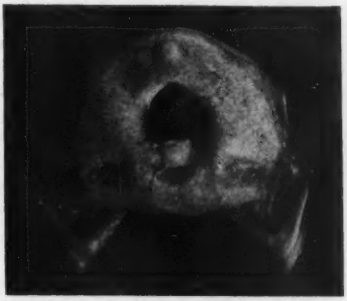

FIGURE 2.—Posterior view of a skull showing the foramen magnum.

Class	*Males*	*Females*
American	1.20	1.19
Asiatic	1.31	1.17
English	1.30	1.27
Mediterranean	1.28	1.16

In the above calculations, individual ratios of less than 1 were eliminated, and these were usually found in females. The foregoing data, together with the observation that ratios of less than 1 were usually those of females, would indicate that the foramen magnum in females is proportionally narrower than in males. Though the differences were slight, the mean female ratio in each class was less than that of the males. It also cannot be said that the Asiatics differed markedly from the other classes. Thus the results found here would not support the statement of Tegetmeier that the Asiatics, as a group, differ significantly from other classes with regard to the relative dimensions of the foramen magnum (occipital foramen).

DISCUSSION

The observations of Tegetmeier, which he considered as evidence of polyphyletic origin of the breeds of fowl, though interesting, probably throw little light on the path of origin of the domesticated fowl. Evolutionists have long since abandoned the idea that similarities of structure between domesticated and wild species are critical evidence

as to the line of descent. The fact that the wild Indian jungle fowl has so many characteristics in common with some of our domesticated forms may indicate only that both have a relatively recent common ancestor.

The recorded history of poultry breeding procedures during the past century, in which most of our present-day breeds and varieties originated, indicates that there was little system or forethought involved. Any group of birds that appeared sufficiently different from existing breeds or varieties to permit identification was admitted to the official list, regardless of its qualities. It is interesting that despite this fact the Mediterranean class of chickens has such a distinctive set of traits, many of which were not set up as breed and varietal specifications. Small body size was a requirement, but other traits such as nervous temperament, large comb, white-shelled eggs, early feathering, and early sexual maturity were never prescribed characteristics of the breeds included in the Mediterranean class. It was earlier thought that there might be some physiological correlation of this group of traits found in the Mediterranean class; but more recent breeding accomplishments have demonstrated that at least early feathering, early maturity, and the white shell color may be combined with large body size. The observation here recorded that some of the varieties of the Mediterranean group have distinctive skull structures further supports the earlier evidence that this group of chickens has a genetic constitution which may have been drawn from an isolated pool of germ plasm.

It is not here contended that this evidence has any bearing on the early evolution of the domesticated chicken, but it may indicate something regarding the derivation of the more recent types of fowl. It is known that the earlier members of the Mediterranean class had their origin in Italy, while the foundation stock for our present day Asiatic breeds had its origin in importations from China and India. The early types were known as Cochin Chinas, Chittagons, Shanghais, and Brahma-pootras. The existing representatives of the American and English classes are known to have come largely from the early Asiatic importations, and these two classes have no special significance except to indicate source of origin. The lack of many of the traits of the Mediterranean group might be taken to indicate that there was not much infusion from this source into the constitution of the American and English classes. This view is further supported by the fact that the white varieties of the larger breeds are due to a recessive gene while the white of the Leghorn is due to a dominant gene.

The fact that the data in this paper do not support the statements of Tegetmeier does not necessarily mean that his observations were incorrect, since the stocks from the Asiatic group here examined were the results of approximately 60 years of selection beyond those available to Tegetmeier. It is of interest to note that the Mediterranean and Asiatic classes showed rather widely differing structure of the frontal bones of the skull when such differences were not observable in the living bird and could not have been intentionally modified by selection. Furthermore, the American and English groups were intermediate between the Asiatics and the Mediterraneans with regard to the structure of the frontal bones. The American and English classes are known to be of more recent origin than the Mediterranean and Asiatic classes and probably had their origin in stocks of these earlier established breeds.

SUMMARY

From these studies it is evident that there are rather wide differences in the structure of the skull at the point of union of the two frontal bones of breeds and varieties of poultry. The suture between the two frontals may be virtually smooth, sharply grooved, or ridged. There is also considerable variation in the depth of the groove. The representatives of the Asiatic and Mediterranean classes here studied showed considerable difference in the range of variation. Of the five classifications, extremely grooved, moderately grooved, slightly grooved, flat, and ridged, the Mediterraneans showed all types but relatively little grooving of any degree. The Mediterraneans had considerable ridging of the skull. The Asiatic group possessed no ridge but were distinguished by a high incidence of grooves, some of which was extremely deep. Although there was considerable overlapping of types of the two groups, they do show distinctive ranges of types. The Mediterraneans have relatively larger combs, but this would hardly seem to be responsible for the structural differences in the skull. No observed differences in skull structure lend support to the contention that the Mediterranean group is basically different in its origin from other groups. This study did not reveal any obvious differences in the structure of the foramen magnum as reported by Tegetmeier.

EFFECT OF INOCULATING A SOIL WITH AZOTOBACTER UPON PLANT GROWTH AND NITROGEN BALANCE [1]

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INTRODUCTION

In 1923 a 20-year experiment was begun in an effort to gain information on the following points: (1) The factors that influence the longevity of *Azotobacter* when introduced into a soil from which it is normally absent; (2) the effect of introducing and maintaining an *Azotobacter* flora in such a soil upon the crop-producing ability and the nitrogen balance of the soil.

The recent comprehensive survey of the literature on this subject by Allison [2] eliminates the necessity of a review of previous work; those interested in the problem are referred to this excellent summary.

METHODS

A nearly level and apparently uniform area of Geary silt loam, about 30 by 60 feet, lying at the summit of a gentle slope was selected for the experiment. The pH of the cultivated layer of this soil was in the vicinity of 5.8, and repeated tests for *Azotobacter* failed to reveal its presence.

Thirty-two 20-gauge sheet-metal cylinders, 3 feet long with a cross-sectional area of 5 square feet, were placed on the area, 6 feet apart from center to center in 4 rows east-west and 8 rows north-south. Prior to installation the cylinders were coated with asphalt paint and numbered from south to north, beginning at the southeastern corner. The soil was removed from an area slightly larger than the cylinder in four 8-inch layers and the cylinders were sunk into the soil 32 inches. Then each layer of soil, after being thoroughly stirred, was replaced in its original position.

Three months after installation the soil in the various cylinders received the following treatments, after which it was thoroughly spaded to a depth of 8 inches:

Series	*Cylinders No.*	*Treatment*
1	1 and 22	glucose.
2	2, 12, 18, and 28	inoculation with *Azotobacter*.
3	3, 9, 19, and 25	lime.
4	4, 10, 20, and 26	lime and inoculation with *Azotobacter*.
5	5, 15, 21, and 31	glucose, lime, and inoculation with *Azotobacter*.
6	6, 16, 17, and 27	no treatment (controls).
7	7, 13, 23, and 29	straw, lime, and inoculation with *Azotobacter*.
8	8, 14, 24, and 30	lime, inoculation with *Azotobacter*, and fallowed.
9	11 and 32	straw.

[1] Received for publication July 6, 1948. Contribution No. 237, Department of Bacteriology, Kansas Agricultural Experiment Station.

[2] ALLISON, F. E. AZOTOBACTER INOCULATION OF CROPS: I. HISTORICAL. Soil Sci. 64: 413–429. 1947.

The straw (wheat), glucose (commercial), and lime (precipitated $CaCO_2$) were added at the rate of 2,000 pounds per acre. The inoculum consisted of 100 gm. of a mixture of several soils known to be abundantly supplied with *Azotobacter* naturally and, in addition, heavily inoculated with a mixture of crude laboratory cultures of *Azotobacter* obtained from a number of soils. The straw and glucose treatments were repeated annually following harvest, at which time all stubble and weeds were spaded into the soil.

Tests for available phosphorus, conducted on soil subsequent to treatment, indicated a possible deficiency. In 1925 and again in 1927 each cylinder received an application of superphosphate at the rate of 500 pounds per acre. In 1932 a second application of lime was given all soils receiving an initial lime treatment. At the same time all limed soils which received an initial inoculation with *Azotobacter* were reinoculated. The effect of this inoculation is evident in cylinder No. 6 (see table 1), which through error received the treatment intended for cylinder No. 5. Because of this error the data for cylinder 6 are not included in any calculations.

All cylinders and the surrounding area were seeded annually to a grain crop. The young plants in the cylinders of series 8 were pulled and spaded into the soil. Winter wheat was planted during the first few years, but it was found that a more uniform stand of oats could be obtained, and thereafter, with the exception of the final crop, oats were planted. Millet was planted the last season in the hope that any variations in the available nitrogen content resulting from treatment might be more strikingly reflected in the growth of a heavier feeding crop.

The crop was harvested by cutting just above ground and air-dried, and if the formation of grain seemed to justify its separation, the weight of seed as well as total weight was recorded. In some years the crop was so nearly a complete failure or suffered such serious damage from insects or rodents before harvest that yields were regarded as unsatisfactory for comparison. In such cases the crop was spaded into the soil. The total weight of 16 crops was regarded as satisfactory for comparative purposes; of these the grain weights of 12 were recorded separately.

On 15 occasions during the 20 years of the experiment the soil was sampled and tested for the presence of *Azotobacter*. Tests of numerous samples gave assurance that the pH value remained approximately 6.5 in the limed soils.

At the beginning and at the end of the experiment the soil of each cylinder was sampled in 8-inch sections for chemical analysis. The samples collected in 1923 were stored, and following completion of the experiment all samples were analyzed for total nitrogen by the Gunning method.

In cultivating the soil and in the collection of samples, those soils were handled first which, because of treatment or past observations, were regarded as being less likely to contain *Azotobacter*. This was the only precaution taken to prevent the transfer of *Azotobacter* from one cylinder to another.

During the first 10 years of the experiment, tests for *Azotobacter* were carried out by inoculating 50 ml. of a modified Ashby solution in a 300-ml. Erlenmeyer flask with the equivalent of 5 gm. of soil, incubating cultures at 28° C., and observing for the development of an *Azotobacter* film. This method was satisfactory for differentiating between the presence and absence of *Azotobacter* but gave little indication as to the relative number present. Beginning with 1933, the same medium solidified with washed agar was poured into petri dishes and one-half gram of soil spread over the surface. The plates were incubated at 28° C. and observed for the development of *Azotobacter* colonies. The data recorded in table 2 for dates prior to 1933 are, therefore, not directly comparable with the data for later dates.

RESULTS

Data relative to the presence of *Azotobacter* in the variously treated soils throughout the experiment are recorded in table 1. Attention has been called to a difference in the method of testing for *Azotobacter* during the first and last halves of the experimental period. This difference somewhat complicates a comparison of the data; nevertheless certain facts are clearly indicated:

(1) At no time did the uninoculated soil contain appreciable numbers of *Azotobacter*.

(2) When *Azotobacter* alone was added to the soil it could be detected for a short period of time, after which it completely disappeared. (3) The application of phosphate and glucose or phosphate and wheat straw had no perceptible influence upon the longevity of introduced *Azotobacter*. (4) The application of lime alone so altered the soil as to make it capable of supporting *Azotobacter* when this organism was added (series 4) or accidentally gained entrance (compare cylinders 3 and 19 of series 3 with Nos. 9 and 25). Attention might be called to the relatively slow transfer of organisms from cylinder to cylinder; the soil in cylinders 3 and 19, for instance, had not become infected with *Azotobacter* after 20 years. (5) The addition of wheat straw or glucose, particularly the latter, along with phosphorus and lime made the soil a more favorable habitat for *Azotobacter* than did the lime alone. (6) The fallowed soil (series 8) served as a somewhat more favorable medium than a similarly treated cropped soil (series 4), possibly because of a more favorable moisture content.

Table 2 presents a summary of the data obtained relative to the influence of the various treatments upon the pH of the soil, the yields, and the nitrogen balance. It should be pointed out in connection with these data that in both yields and changes in the nitrogen content of the soil, differences between the cylinders in a given treatment were in many instances greater than the differences between the means of different treatments; hence no great significance can be attached to the recorded mean differences.

These data reveal that the pH of soils receiving no lime was between 5.6 and 5.9, and that all limed soils were maintained at a pH of approximately 6.5 throughout the experiment. Treatments other than liming resulted in no significant alteration in pH value.

TABLE 1.—*Effect of various treatments of an Azotobacter—free soil upon the longevity of introduced Azotobacter*

Year and cylinder No.	Relative abundance of *Azotobacter* [1] in cylinder No. —																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1924	−	4+	−	4+	4+	−	4+	4+	−	4+	−	4+	4+	4+	4+	−	−	4+	−	4+	4+	−	4+	4+	−	4+	−	4+	4+	4+	4+	−
1925	−	4+	1+	4+	4+	−	4+	4+	?	4+	−	4+	4+	4+	4+	−	−	4+	−	4+	4+	−	4+	4+	1+	4+	−	4+	4+	4+	4+	−
1927	−	−	−	4+	4+	−	4+	4+	?	4+	−	−	4+	4+	4+	−	−	?	−	4+	4+	−	4+	4+	?	4+	−	−	4+	4+	4+	−
1928	−	−	−	4+	4+	−	4+	4+	−	4+	−	?	4+	4+	4+	−	−	−	−	4+	4+	−	4+	4+	−	4+	−	−	4+	4+	4+	−
1930	−	−	−	4+	4+	−	4+	±	−	4+	−	−	4+	4+	4+	−	−	−	−	4+	4+	−	4+	4+	−	4+	−	−	4+	4+	3+	−
1933	−	−	−	4+	2+	[2]4+	2+	1+	−	2+	−	−	2+	2+	4+	−	−	−	−	2+	4+	−	2+	2+	−	2+	±	?	2+	3+	4+	−
1935	−	−	±	1+	3+	....	3+	2+	−	1+	−	−	±	3+	4+	−	−	−	−	1+	4+	−	±	2+	−	1+	−	−	1+	2+	4+	−
1936	−	−	−	−	4+	....	2+	2+	−	1+	−	−	1+	3+	4+	−	−	−	−	2+	2+	−	2+	2+	2+	2+	−	−	....	2+	4+	−
1937	−	−	−	2+	4+	....	3+	2+	1+	−	−	−	2+	4+	4+	−	−	−	−	1+	4+	−	1+	2+	2+	2+	−	−	1+	2+	4+	−
1938	−	1+	−	±	4+	....	4+	4+	±	−	−	−	2+	2+	4+	−	−	−	−	2+	4+	−	2+	3+	2+	2+	−	−	2+	4+	4+	−
1938	−	−	−	−	2+	....	4+	2+	−	1+	−	−	....	4+	4+	1+	−	1+	1+	2+	4+	1+	1+	2+	2+	1+	−	−	1+	2+	4+	±
1939	−	....	−	−	4+	....	2+	2+	2+	−	−	−	2+	4+	4+	−	−	−	−	2+	4+	−	2+	2+	2+	2+	−	−	2+	2+	4+	−
1940	−	−	−	2+	4+	....	4+	2+	1+	1+	−	−	2+	4+	4+	−	−	−	−	1+	4+	−	1+	3+	2+	2+	1+	−	2+	2+	4+	−
1941	−	−	−	2+	4+	....	4+	2+	2+	2+	−	−	2+	3+	4+	−	−	−	−	−	4+	−	±	3+	2+	3+	−	−	2+	3+	4+	−
1943	−	−	−	±	4+	....	−	2+	−	−	−	−	−	2+	4+	−	−	−	−	−	4+	−	−	2+	2+	3+	−	−	2+	2+	4+	−
Mean pH	5.79	5.72	6.50	6.66	6.79	....	6.62	6.48	6.52	6.58	5.94	5.75	6.60	6.57	6.72	5.77	5.78	5.77	6.57	6.58	6.60	5.90	6.61	6.49	6.39	6.63	5.87	5.80	6.62	6.54	6.66	5.83

[1] The relative number of *Azotobacter* is indicated as follows: − =None; ?=indefinite; 1+ =few; 2+ =some; 3+ =many; 4+ =abundant; ± =one of duplicate cultures negative.
[2] This cylinder of soil was inoculated through error just prior to this sampling.

TABLE 2.—*Effect of various treatments upon the pH, Azotobacter content, plant growth in and nitrogen balance of an Azotobacter-free Gary silt loam soil*

Series	Cylinders No.	Treatment	pH	Azotobacter content	Yield, dry weight		Change in nitrogen pounds
					Total	Grain	
					Grams	*Grams*	*Per acre*
6	16–17–27	None	5.8	None	99	35.9	−453
2	2–12–18–28	Inoculated	5.6	do	107	38.8	[1]−88(208)
3	3–9–19–25	Lime	6.5	Few toward end of experiment.	107	39.9	−176
4	4–10–20–26	Inoculated, lime	6.6	Few	105	38.3	[1]−557(403)
1	1–22	Glucose	5.8	None	101	34.6	−390
5	5–15–21–31	Inoculated, lime, glucose	6.7	Abundant	102	34.5	−208
9	11–32	Straw	5.9	None	116	40.2	+219
7	7–13–23–29	Inoculated, lime, straw	6.6	Some	117	42.4	+10
8	8–14–24–30	Inoculated, lime, fallow	6.5	Many			+29

[1] The values in parentheses are the means of the series when a single cylinder that deviated widely from the others is omitted.

The variability in plant growth from year to year and from cylinder to cylinder within the same treatment for any given year is apparent from the fact that the highest annual yield for any single cylinder occurred at least once and not more than four times for every treatment, including the control. Also, both the highest and lowest mean annual yields for any treatment occurred at least once in all but one treatment, and not more than four times for any single treatment. So variable were the yield data that when they were tested statistically the only treatment showing a significant increase over the control at the 5-percent level was the lime-straw-inoculated series. There is no evidence that this increase resulted from the inoculation, and probably should be attributed to nutrients, chiefly nitrogen, added in the straw during the 20-year period.

A somewhat comparable variability in the nitrogen balance for the 20-year period was also recorded. When these data were tested statistically the only treatment showing a significant loss at the 5-percent level was the control series. Despite the absence of statistically significant losses, the fact that the recorded data for the fallow and the two straw-treated series indicated no losses while the recorded data for all other treatments did indicate losses, seems highly suggestive. The fallow soil from which no crop was removed and on which erosion was prevented would not be expected to lose much nitrogen under the relatively low rainfall of this region, while the addition of the limited nitrogen in the straw would, in part, compensate for the nitrogen removed in the crop taken off such treated soils.

Another approach to the influence of the various treatments upon the nitrogen balance can be made by comparing the mean recorded gain or loss in nitrogen of one treatment with that of another to determine whether the difference between the two is significant. Since there were eight treatments exclusive of the fallowed series, which for obvious reasons is not comparable to the others, 28 such comparisons are possible.

The data shown in table 2 suggested that the differences between the mean change in the nitrogen content of treatments involving the application of straw (series 7 and 9) and treatments not including straw (series 1, 2, 3, 4, 5, and 6) would more likely be significant. When a statistical study was made of each of the 16 possible comparisons between 2 straw-treated series or between 2 treatments not including straw, in no instance was the difference between the mean recorded changes in the nitrogen content of the soils found to be significant at the 5-percent level. On the other hand, a similar comparison between straw-treated and non-straw-treated soils revealed that the differences were significant at the 5-percent level in 9 of the 12 possible comparisons, 3 of which were significant at the 1-percent level.

Two of the three comparisons between the straw vs. nonstraw treatments in which differences were not statistically significant involved the series (2) receiving inoculation alone. . This might be interpreted as indicating an influence of the inoculum. However, the mere fact that the *Azotobacter* completely disappeared from the unlimed inoculated cylinders (series 2) in such a short period of time precludes the possibility of its exercising any influence upon the nitrogen balance of the soil over the 20-year period. It may be noted that the mean recorded loss of nitrogen from the inoculated series (2), was only 88 pounds, the low value being attributable to the change in one of the four cylinders deviating so markedly from that of the other three. If this cylinder were left out of consideration the mean loss would be 208 pounds. Similarly, the unusually high recorded loss of 557 pounds for the inoculated-limed series (4), was attributable to a single cylinder, which if left out of consideration, would reduce the mean loss in this instance to 403 pounds. No explanation is available as to why these two cylinders were so far out of line with others similarly treated. Their omission from consideration would bring these two treatments more nearly into line with other somewhat similar treatments.

In the preceding paragraph attention has been called to the virtual impossibility of the *Azotobacter* introduced into the soil of series 2 having any influence upon the nitrogen balance of the soil; thus this series might be regarded as a duplicate of the control series. This being true, the data recorded in table 2 relative to yields indicate that the application of straw was the only treatment giving even a suggestion of any influence of treatment upon yield. Actually the only instance in which any single treatment gave a significant increase at the 5 percent level over any other treatment was the inoculated-limed-straw series (7) over the control series (6). Again, this difference cannot be attributed to the presence of *Azotobacter*, since the application of straw alone (series 9) gave practically the same yield.

SUMMARY

The addition of superphosphate at the rate of 1,000 pounds per acre, or of the superphosphate and glucose or wheat straw at the rate of 2,000 pounds per acre annually, did not influence the longevity of *Azotobacter* introduced into a Geary silt loam, of pH value below

6.0, from which it was absent. The addition of superphosphate and lime adequate to maintain a pH level of 6.5 enabled introduced *Azotobacter* to survive throughout the 20 years of the experiment. The suitability of this soil after liming as a habitat for *Azotobacter* was greatly enhanced by the annual application of a suitable carbohydrate food, i. e., glucose. No evidence was obtained that the maintenance of *Azotobacter* in this soil for a period of 20 years influenced either the crop grown thereon or the nitrogen balance of the soil. The annual application of 2,000 pounds of wheat straw per acre did apparently influence favorably both plant growth and the nitrogen balance of the soil, but this cannot be attributed to the activity of *Azotobacter*.

THE EFFECT OF SUN, SHADE, AND OVEN-DRYING ON THE TOXICOLOGICAL AND CHEMICAL VALUES OF DERRIS ROOT[1]

By Caleb Pagán, Richard H. Hageman, *chemists*, and Arnaud J. Loustalot, *plant physiologist and assistant director, Federal Experiment Station, Office of Experiment Stations, United States Department of Agriculture, Mayagüez, P. R.*

INTRODUCTION

Jones and Pagán (*3*) [2] recently demonstrated that there was no loss of rotenone when fresh derris roots (split or whole) were dried either in the sun, in shade, or in the oven. However, Jones et al. (*4*) and others (*6*, *7*) have also shown that the toxicological value (rotenone equivalent) of derris roots is not perfectly correlated with the rotenone content. The data from these papers indicate that substances other than rotenone contributed to the toxicity of the roots. The fact that there was no loss of rotenone when derris roots were dried under the above condition does not indicate that there may be no loss in toxicity. A quick simple method for biologically determining the toxicity of derris roots has been developed by Pagán (*5*). Guppies (*Lebistes reticulatus* Peters) are used as test animals in this method and the toxicity assays are made in a relatively short time.

The guppy test has provided a convenient method for measuring the effect of sun, shade, and oven-drying on the toxicological value of derris roots. In this experiment various chemical measurements of toxicity including the red color value described by Jones (*2*), total chloroform extractives, and percent transmittance of acetone extracts suggested by Pagán and Loustalot (*6*, *7*) were compared with the toxicological value (rotenone equivalent) of the roots dried in the sun, in the shade, and in the oven.

MATERIALS AND METHODS

The roots used in this experiment were from *Derris elliptica* (Wall.) Benth. variety Sarawak Creeping and were harvested from plants about 5 years old. Approximately 4,000 grams of washed fresh roots 4 to 10 millimeters in diameter were cut into 2- to 2.5-centimeter pieces. After cutting, the entire lot was thoroughly mixed and duplicate 300-gram samples weighed into tared containers and submitted to the following drying treatments until constant weight was obtained: (1) Whole roots were dried immediately in an air oven at 80° C. for 1 hour, then at 50° C. for 4 hours, after which the samples were placed in a desiccator for 4 hours and weighed, alternate drying in the oven

[1] Received for publication January 3, 1949.

[2] Italic numbers in parentheses refer to Literature Cited, p. 416.

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and the dessicator being repeated until constant weighing was obtained; (2) whole roots were dried in the sun; (3) root pieces were split longitudinally and dried in the sun; (4) whole roots were dried in the shade; and (5) split roots were dried in the shade. Samples of fresh root were taken at the outset and dried immediately at 105° to determine the initial moisture content.

When the roots in all treatments had reached constant weight subsamples were taken and dried at 105° C. to determine the final moisture content. They were then ground in a Wiley mill to pass an 80-mesh sieve and the toxicological and chemical values of the powdered roots determined. The rotenone content was determined by the method of the Association of Official Agricultural Chemists (*1*) and the chloroform extractives were determined from an aliquot of the chloroform extractives prepared for rotenone analysis. This aliquot was evaporated to dryness on a water bath and then in an oven at 105° C. for half-hour periods alternated with cooling in a desiccator over $CaCl_2$, until constant weight was attained. The red color (or rotenone plus rotenoids) value was determined by the modified red color test described by Jones (*2*). The transmittance value of the acetone extract of the roots was determined by the method described by Pagán and Loustalot (*6*), and the rotenone equivalent was determined with guppies as described by Pagán (*5*) Five replications of each treatment were used in the biological assays and the results were analyzed statistically.

RESULTS AND DISCUSSION

The roots dried in the oven reached constant weight within 1 day. Four days were required to dry whole roots in the sun in contrast to 6 days required for drying them in the shade. Sun-drying of split pieces was completed in 2 days, while 4 days were required to dry them in the shade. Weather conditions during the experimental period were more favorable for drying than in the previous experiment (*3*). The data presented in table 1 show that there was no loss of dry matter in whole roots dried in the oven or in whole or split roots dried in the sun. However, there was a small loss of dry matter in roots dried in the shade. This loss was somewhat greater in the whole roots which required 2 days longer to dry than the split roots. The losses in dry

TABLE 1.—*Effect of sun, shade, and oven-drying on the toxicological and chemical values of derris roots*

Drying treatment	Dry-matter losses	Rotenone	Toxicological value as rotenone equivalent [1]	Red color value	Total chloroform extract	Transmittance values of acetone extract at 300 mμ [2]
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Whole roots, dried in oven	None	6.4	11.3	14.6	18.9	34.5
Whole roots, dried in sun	do	6.3	13.5	14.7	18.5	34.2
Whole roots, dried in shade	2.1	6.3	16.9	14.9	18.8	33.6
Split roots, dried in sun	None	6.3	16.4	15.0	19.9	32.8
Split roots, dried in shade	.9	6.4	16.3	14.9	19.9	33.6

[1] The toxicological rotenone equivalent is the percentage of rotenone the root would need to contain in order to give the kill actually found.
[2] High values indicate low toxicity and vice versa.

matter are probably due to metabolic activity as suggested in the previous drying experiment (*3*).

The rotenone content of the roots was practically the same for all treatments. These results are in agreement with those reported by Jones and Pagán (*3*). The results of the biological assays also shown in table 1 revealed that the derris roots dried in the sun or shade, either split or whole, had a significantly higher toxicity expressed as rotenone equivalent than those oven-dried. Since the rotenone content of all samples was essentially the same, these data would indicate that some substance that adds to the toxicity of the root, other than rotenone, was lost or changed chemically during the period of oven-drying. Although the rotenone equivalent of whole roots dried in the sun was somewhat lower than that of split sun-dried roots or of whole and split roots dried in the shade, the difference was not statistically significant.

The red-color values were not markedly different in the roots of the various treatments, although there was some tendency for these values to be higher when the rotenone equivalent was high.

There was no statistically significant deviation in the total chloroform extractives of the various treatments. It is of interest to note that the percentage total chloroform extractives is greater in the roots that were split irrespective of sun or shade drying than in those of the other treatments. This may be due to the fact that the additional surface exposure of the fresh root produced by splitting afforded more opportunity for air oxidation of the root constituents. The percentage transmittance of acetone extracts of the roots correlate with the toxicity of the roots, much better than any of the other chemical assays.

SUMMARY

1. Fresh derris roots whole and split were dried in the sun, in shade, and in the oven until constant weight was attained. Whole roots in the oven were dried in less than 1 day. Whole roots dried in the sun required 4 days. Split roots were sun dried in 2 days while 4 days were required to dry them in the shade.

2. The rotenone content of roots of all treatments was practically the same, indicating that the drying treatments had no effect on this toxic constituent.

3. There was no statistically significant difference between the toxicological value of the roots dried in the sun or shade, whole or split. However, the rotenone equivalent of roots dried in the oven was significantly lower than those dried in the sun and shade. The fact that the rotenone content was the same in all roots indicates that some other constituents that contribute to the toxicity was destroyed or changed by the treatment.

4. The red color values were not appreciably different in any of the treatments, although there was a tendency for them to be somewhat higher when the rotenone equivalent was high. As found in the previous experiment, there was no statistically significant deviation in the total chloroform extractives of the roots from the various treatments. The percentage transmittance values of acetone extract of the roots gave the best correlation with the toxicological values.

5. These results indicate that derris roots may be dried either in sun or shade without appreciable loss in toxicity, but that drying

them in an air oven at 80° C., then at 50° C. as in this experiment, results in a lower toxicological value even though there was no loss of rotenone.

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EFFECT OF ROOT DIAMETER ON CHEMICAL AND BIOTOXIC CONSTITUENTS OF DERRIS [1]

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INTRODUCTION

The literature shows that there are different opinions as to the relationship between diameters and toxicological values of *Derris* roots. Moreau (*8*) [2] and Sievers (*12*) state that the large roots are generally inferior in quality as determined by their total extractives and rotenone content. In contrast, Worsley (*14*) reports an average rotenone content of 9.41 percent in "coarse" roots and 7.49 percent in fine roots. Koolhaas (*5*) in Java found that the extremely fine roots (1- to 2-mm. diameter) contained 9.0 percent rotenone while the large roots (10- to 17-mm. diameter) had but 4 percent. Moreau (*8*) states that in East Africa it has not been necessary to reject any of the roots from 3-year-old plants. This implies that even the large roots contained sufficient rotenone (5 percent) to be marketable.

Most workers in the field, however, believe that the medium roots, 4- to 10-mm. in diameter, are superior in rotenone content to the fine or large roots. The chemical data presented by different workers seem to support this point of view. The data reported by Maas (*6*) is typical. He found the following relationship between rotenone content and root diameter: Fine roots (below 2-mm. diameter) contained 5.3 percent rotenone, medium roots (2- to 6-mm.) 9.1 percent, and large roots (6- to 13-mm.) 7.9 percent. Moore (*7*) concluded that the medium root was superior in rotenone and total extractives. He found that in general the starch-bearing tissue predominated in the thick older roots and that the resin-cell tissue that contains the rotenoids was relatively more abundant in roots of small and medium diameters.

Since previous evaluations were based on the amount of rotenone or total extractives that the roots contained, it seemed desirable to assay the various root diameters biologically (*9*) and to compare these values with various chemical criteria.

[1] Received for publication January 3, 1949.

[2] Italic numbers in parentheses refer to Literature Cited, p. 422.

METHODS AND PRESENTATION OF DATA

Two commercially important varieties of *Derris elliptica* (Wall.) Benth., Sarawak Creeping, and the MG clones (*4*) of the Changi variety, were studied in this experiment. Two replicates of 25 plants for each variety were harvested from border rows of a previous experiment. The Sarawak Creeping plants were 48 months old at time of harvest and the Changi-Mg clones 36 months. Because of this difference in age at harvest direct comparison between the two varieties cannot be made. The plants were excavated as completely as possible and the fresh roots were divided into the following diameter groups: 2 mm. or less, 2 to 4, 4 to 10, and over 10 mm. Many of the thick roots in the Sarawak Creeping variety were over 25 mm., which is much larger than ordinarily found in roots harvested at 26 to 32 months. In the MG clones the maximum root diameter was around 15 mm., which is normal. All the roots were dried in the sun; the thicker roots were split to hasten drying. The air-dry weights of the different sizes in each variety are given in table 1.

TABLE 1.—*The yield of roots and their toxic constituents obtained by a diameter classification of 2 varieties of Derries elliptica, MG and Sarawak Creeping (50 plants each)*

	Yield of roots		Yield of rotenone [1]		Yield of rotenone equivalent [2]	
MG clones:	*Grams*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent of total*
Less than 2 mm	165.0	4.4	13.5	4.5	14.2	4.1
2–4 mm	447.0	12.0	49.2	16.6	54.9	15.6
4–10 mm	2,299.0	61.9	197.7	66.5	241.0	69.1
10+ mm	802.0	21.7	36.9	12.4	39.3	11.2
Total	3,713.0		297.3	[3] 8.0	349.4	[4] 9.4
Sarawak Creeping:						
Less than 2 mm	408.5	6.4	15.1	6.0	22.4	6.7
2–4 mm	1,423.5	17.6	65.2	25.9	90.5	27.0
4–10 mm	2,472.0	38.7	133.5	53.0	222.6	66.3
10+ mm	2,385.0	37.3	38.2	15.1	0	0
Total	6,389.0		252.0	[3] 3.9	335.5	[4] 5.3

[1] Rotenone yield obtained by multiplying the percentage of rotenone determined chemically by the yield of root (gram) of each of the 4 diameter sizes.
[2] Rotenone equivalent yield determined by multiplying the percentage rotenone equivalent obtained by the guppy test by the yield of root (gram) of each of the 4 diameter sizes.
[3] Obtained by dividing total rotenone in grams by total yield of roots (gram).
[4] Obtained by dividing total rotenone equivalent in grams by total yield of roots (gram).

In the MG clones the bulk of the root, about 62 percent, consisted of medium roots of 4 to 10 mm. in diameter. The rest of the yield was distributed as follows: 4 percent in the 0- to 2-mm. group, 12 percent were 2 to 4 mm., and 22 percent were over 10 mm. in diameter. The air-dry yield per plant was 74.2 gm. and the total yield for the 50 plants 3,713 gm.

The distribution by diameter groups of the Sarawak Creeping roots followed an entirely different pattern. Roots of the 0- to 2-mm. size yielded 6 percent of the total, while 17 percent were found to be in the 2- to 4-mm. class. The two largest diameter groups, 4 to 10 and 10+ mm. yielded 38 and 37 percent, respectively. These data agree fairly well with the results obtained by White, Pagan, and Jones (*13*). The yield per plant in the Sarawak Creeping variety was 127.7 gm. and the total yield for the 50 plants 6,389 gm.

The roots were all ground to pass a 0.5-mm. sieve and analyses were made for rotenone (*1*), red-color value (*2*), total chloroform extractives, and transmittance (*10*). The results from these determinations are presented in table 2. The biological assay was made by the "guppy" method (*9*). In order to facilitate statistical analysis between the different root sizes, the same amount of root powder (0.250 mg.) was weighed in all cases. A composite sample of all roots of each of the varieties was included in the tests. These composites were made by mixing the different root diameters on the basis of weight percentages of their respective yield of air-dry roots. The toxicological data is presented in table 3.

TABLE 2.—*Chemical analyses by root diameters of 2 varieties of Derris*

Derris elliptica	Root diameter	Rotenone [1]	Total $CHCl_3$ extractives [2]	Red color value [3]	Transmittance
	Millimeters	*Percent*	*Percent*	*Percent*	*Percent*
MG clone	0-2	8.2	19.5	17.5	52.5
	2-4	11.0	26.5	23.5	48.5
	4-10	8.6	20.3	18.1	56.0
	10+	4.6	10.2	9.0	70.5
	Composite	7.0	17.3	15.0	53.0
Sarawak Creeping	0-2	3.7	10.6	9.5	63.0
	2-4	5.8	15.3	14.0	57.5
	4-10	5.4	15.7	12.5	58.0
	10+	1.6	5.2	4.6	79.0
	Composite	4.1	10.9	10.0	61.0

[1] Differences between rotenone percentages greater than 0.89 percent are significant at odds of 99:1.
[2] Differences between total choloroform extractives percentages greater than 2.6 percent are significant at odds of 99:1.
[3] Differences between red-color value percentages greater than 2.2 percent are significant at odds of 99:1

TABLE 3.—*Results of toxicological assay by root diameters of 2 varieties of Derris*

Variety of *Derris elliptica*	Root diameter	Test solution [1] rotenone	Mean [2] mortality	Standard error	Rotenone equivalent [3]		Increase in toxicity due to rotenoids
					Test solution	Root	
	Millimeters	*Milligrams per milliliter*	*Percent*	*Percent*	*Milligrams per milliliter*	*Percent*	*Percent*
MG clone	0-2	0.082	50.0	4.1	0.086	8.6	4.8
	2-4	.110	72.5	2.5	.122	12.2	10.9
	4-10	.086	62.5	6.3	.105	10.5	22.0
	10+	.046	20.0	7.1	.049	4.9	6.5
	Composite [4]	.070	67.5	11.1	.112	11.2	59.9
Sarawak Creeping	0-2	.037	22.5	8.5	.055	5.5	48.6
	2-4	.058	47.5	7.5	.081	8.1	39.6
	4-10	.054	55.0	5.0	.090	9.0	66.5
	10+	.016	0	0	0	0	0
	Composite [4]	.041	25.0	6.5	.054	5.4	31.7
Rotenone:							
Standard 1		.050	22.5	7.5			
Standard 2		.100	62.5	9.5			
Standard 3		.200	90.0	4.1			

[1] Concentration of root powder in test solution was in all cases 1.0 mg. per ml. of acetone.
[2] Differences between mean mortalities of 9.7 per cent were significant at odds of 19:1.
[3] Rotenone equivalent is the amount of rotenone the root must contain in order to give the kill actually found.
[4] Compounded on a percentage weight basis to represent the entire root and analyzed separately.

Based on the results of the biological assays, the diameter groups 2 to 4, and 4 to 10 mm. in Sarawak Creeping were the most toxic for this variety and both were superior by high significance to roots less than 2 mm. and those over 10 mm. in diameter. Roots less than 2 mm. in diameter ranked third in toxicity while the largest roots were the least toxic. The thick roots (over 10 mm. in diameter) produced no mortality in the guppy test. The amount of rotenone plus the unknown amount of rotenoids present in the 250-mg. sample used in the biological tests did not give an extract of sufficient concentration to reach the toxicity threshold. A toxicity value could have been established for the large diameter roots by increasing the amount of powder used for the test. This was not done because the statistical treatment of the data required a constant weight of sample for each of the diameter groups. It is interesting to note that nearly 35 percent of the total yield of dry Sarawak Creeping roots belonged to this group of large diameter roots.

The bio-assay of the MG clones gave results entirely different from those obtained with Sarawak Creeping. Roots with diameters from 2 to 4 mm. were significantly better than the other three groups. Roots in the 4- to 10-mm. class came next, followed closely by 0- to 2-mm. group. These two groups were significantly better than the roots 10 mm. in diameter and larger.

The rotenone equivalent for the composite sample of Sarawak Creeping was found to be 5.4 percent; as shown in table 1 the value calculated from the individual percentage rotenone equivalents of the diameter groups was 5.3 percent. This good agreement between the calculated and the observed toxicity of Sarawak Creeping roots is in sharp contrast with the values found in the MG clones. In this variety the calculated rotenone equivalent was 9.4 percent and the actual value as determined on guppies was 11.2 percent. The reason for this disagreement is not known.

Table 1 also presents a comparison of the yield of rotenone equivalent by diameter groups. The yield was obtained by multiplying the mean yield of air-dry root for each diameter by the percentage of rotenone equivalent obtained with the guppies. These results show that even though the best quality roots of the MG clones were in the 2- to 4-mm. diameter group and the best quality Sarawak Creeping roots were in the 4- to 10-mm. group, the highest yield in both cases fell in the 4- to 10-mm. group.

The relation between chemical and toxicological constituents is shown graphically in figure 1. In this figure the different chemical constituents and the rotenone equivalent were plotted against the root diameter. The similarity of the shape of the lines show that in both Sarawak Creeping and MG clones there is a direct relationship between the chemical values and the actual toxicity. The total chloroform extractives in both varieties correlated more closely with rotenone equivalent than did any of the other chemical constituents. This can be seen also from the ratios presented in table 4 and substantiates experiments previously reported (*11*). The transmittance values obtained from the Sarawak Creeping plants correlate quite well with the bio-assay, while in the MG clones this value does not agree with the rotenone equivalent as well as the other chemical constituents.

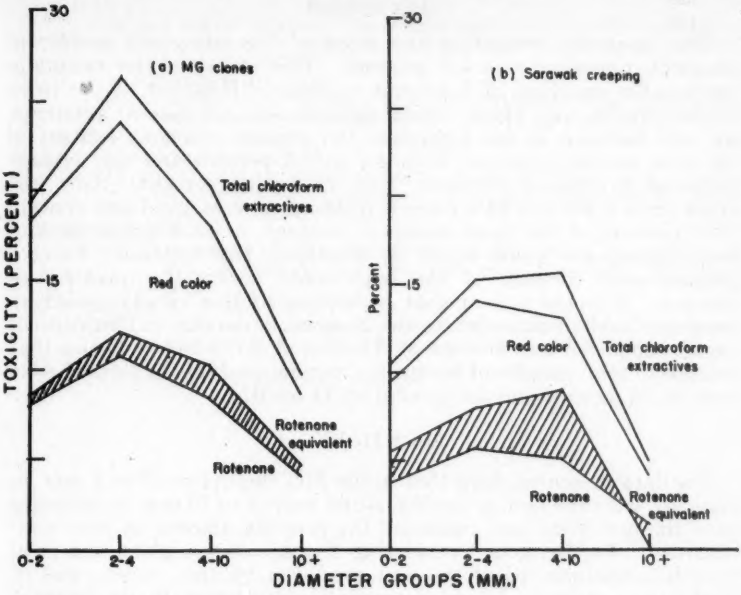

FIGURE 1.—Chemical and toxicological values of some derris roots plotted against root diameters.

TABLE 4.—*Comparison of chemical and toxicological values by diameter groups of 2 varieties of derris root*

Sample	Ratio of rotenone equivalent to—		
	Total $CHCl_3$ extractives	Red color value	Rotenone
MG clones:			
Less than 2 mm	0.440	0.491	1.05
2-4 mm	.460	.519	1.11
4-10 mm	.517	.580	1.22
10+ mm	.480	.544	1.06
Sarawak Creeping:			
Less than 2 mm	.519	.578	1.48
2-4 mm	.529	.578	1.40
4-10 mm	.573	.720	2.00
10+ mm			

The increase in toxicity due to rotenoids is very noticeable in Sarawak Creeping and less so in MG clones, as shown by the shaded area in figure 1. This is in agreement with the work of Jones et al (*3*). In both varieties the rotenoids in 4- to 10-mm. roots were most toxic, causing an increase in toxicity of 66.5 percent in Sarawak Creeping and of 22.0 percent in MG clones.

DISCUSSION

The rotenone content in the roots of the composite sample of Sarawak Creeping was 4.1 percent. This is below the minimum marketable standard of 5 percent rotenone. However, if the thick roots (10-mm. and over), which contain only 1.6 percent rotenone, are not included in the composite the average rotenone content of the root system increases from 4.1 to 5.3 percent and the average rotenone equivalent increases from 5.4 to 8.4 percent. Since the thick roots represent 33.0 percent of the total root yield and contain 15.0 percent of the total rotenone content, a considerable loss of toxic ingredients would result by discarding this material. Since a proportionate increase of the large roots lowers the quantity of rotenone, it would appear that harvesting of this variety would be most profitable if done before the large roots develop. This contention is supported by the work of White et al (*13*) which indicates that composite root samples of Sarawak Creeping contain 6.6 percent rotenone at 26 months and 5.6 percent at 32 months.

SUMMARY

The data presented show that in the MG clones roots 2 to 4 mm. in diameter were the best in quality, while roots 4 to 10 mm. in diameter gave the best yield and contained the greatest amount of toxic constituents. Furthermore, considering the age of the plant and local growth conditions, all of the root produced by this variety was of marketable quality. In the Sarawak Creeping variety the group 4 to 10 mm. in diameter was the best in quality and contained the greatest quantity of toxic constituents. Roots less than 2 mm. or above 10 mm. in diameter do not have the minimum rotenone content for marketable roots; rejecting the thick roots (10 mm. and over) would bring the remaining roots to marketable standard.

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CRITERIA FOR TESTING VANILLA IN RELATION TO KILLING AND CURING METHODS[1]

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INTRODUCTION

The widely known flavor, vanilla, is prepared from the vanilla bean which is the fruit of a tropical orchid, *Vanilla fragrans* (Salisb.) Ames. Actually, the fruit is not a bean but a pod, 5 to 10 inches long, one-quarter to three-quarters of an inch in diameter, and somewhat triangular in cross section. Inside the thick fleshy outer wall are numerous small black seeds. The pods are harvested from the plant when a yellow color develops on the blossom end of the pod. They are then cured in four steps as follows:

(1) Killing—by wilting, or killing by immersion in hot water or by sunning;
(2) Sweating—by exposure to the sun and wrapping in blankets, or warming in an oven;
(3) Drying (the beans now dark brown and flexible) to 15 to 30 percent;
(4) Conditioning—storing the product for several months in closed boxes at room temperature, during which time the flavor develops.

The cured material has the characteristic vanilla aroma and is ready for market.

For use as a flavor, the beans are ground with sugar and other ingredients or extracted with alcohol or some other nontoxic solvent to obtain the well-known vanilla extract. To enhance its flavoring value, the final product is often fortified with vanillin prepared from lignin or from clove oil.

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[1] Received for publication January 3, 1949.

[2] The authors are indebted to Mr. Robert Rosenbaum of David Michael and Company, Philadelphia, Pa., vanilla processors. Mr. Rosenbaum examined various extracts referred to in this paper and furnished written opinions of his results. His tests were based on the aroma of the extracts as they are slowly evaporated without heating.

CRITERIA FOR TESTING VANILLA

It is generally agreed that the criteria usually applied to vanilla do not constitute a true measure of the quality of the product. An analysis of vanillin and determination of phenol value, lead number, and resin content may be useful in identifying vanilla and detecting adulteration, but they are of little value in measuring quality. Even the usual grader's examination for texture, color, aroma, and appearance, which is valuable in obtaining rapid estimation of quality, is not entirely satisfactory. Each of these properties is only symptomatic of quality; that is, the product may appear to be excellent according to several of these criteria and yet may be quite inferior when used as a flavor. Or, two samples may appear fairly equal according to these tests and still differ markedly in flavoring strength and other characteristics.

Vanilla, as well as other similar materials, can be tested organoleptically in ice cream by several testers and the results analyzed statistically to evaluate the degree of difference among the samples. This method was tried on a set of extracts, to evaluate both the products and the techniques of testing.[3]

The same extracts were used numerous times at various strengths and in different recipes. The most satisfactory ice cream recipe to date calls for 1 pint of cream (40-percent butterfat), 4 pints of milk (4-percent butterfat), 15 percent sugar, and 0.3 percent gum tragacanth. The mix was divided into separate portions, one for each sample and the extract added at the rate of 2 cc. per 100 cc. of mix. To one sample in each series, the blank, no extract was added. After cooling to 10° C. the samples were churned in a hand freezer to a hard consistency and stored at −10° until tested.

The vanilla extracts used throughout these curing experiments were prepared as follows: The moisture content of the cured beans was determined so that the extracts could be made with a known proportion of alcohol to dry vanilla tissue. The beans were cut to 1-cm. slices and soaked in 10 cc. of 50-percent alcohol per gram of dry matter. The beans and alcohol were kept in a flask fitted with an air condenser. Over a period of 2 weeks the flasks were warmed twice daily in a water bath at 60° C. At the end of this time, the extracts were filtered through cotton to remove the solids.

The ice cream samples were tasted from 3 to 22 hours after freezing in two series with coded designations. The ranking of each tester was checked for reliability by calculating the correlation coefficient between the two separate rankings made by the same tester. The data were accepted as reliable if the tester obtained the same ranking both times, or if in the second run he interchanged two samples other than the blank that were consecutive in the first run. However, if the tester interchanged first and third rank, the correlation coefficient, then below 0.77, was considered too low and the tester's data were rejected. Using the ranks obtained by reliable testers, the

[3] BLISS, C. I., ANDERSON, E. O., and MARLAND, R. E. A TECHNIQUE FOR TESTING CONSUMER PREFERENCES, WITH SPECIAL REFERENCE TO THE CONSTITUENTS OF ICE CREAM. Connecticut [Storrs] Agr. Expt. Sta. Bul. 251, 20 pp. 1943.

data were then treated statistically as though each ranking were made by a different tester. For example, if three reliable testers each made two acceptable determinations, the final statistical analysis was made as though there were six testers.

The same set of extracts was used on several different dates and scores for the samples calculated each time. If, on any given date, the differences between scores were not considerably greater than that necessary for high significance, the data for that date were discarded on the assumption that the recipe used was such that the differences between samples were not brought out sharply. Of course, if this occurred consistently, the data were accepted and the conclusion drawn that the differences among the samples were not great. However, where appreciable differences were obtained on some dates, the data from runs in which differences were small were discarded and the weakness of the data attributed to the receipe.

COMPARISON OF KILLING METHODS

In 1943 an experiment in which different curing methods were compared showed that scratching the beans was a good way to kill them.[4] Recently, further trials were made with combinations of the following killing methods singly and in combination: (1) Dipping in water at 80° C. three times for 10 seconds at 30-second intervals; (2) scratching each face 1 mm. deep from end to end (some beans were scratched only 2 cm. from the stem end to decrease the brittle nature of the stem end in the cured product); and (3) frozen at −10° for 24 hours. After killing the samples were oven-sweated at 45° and dried at room temperature to 28.6 percent of the fresh weight, after which they were conditioned in closed boxes at room temperature for 6 months. Duplicate 260-gm. samples of 6- to 8-inch blossom-end-yellow beans were used. No mold developed in any of the samples and splitting was negligible.

Those killed with hot water were smooth, oily, and dark brown during sweating. During drying the oiliness changed to stickiness and a very slight vanilla aroma became noticeable. The conditioned product was very dark brown, somewhat rough, and not flaccid. The beans were shiny and had no vanillin crystals on the outside. The aroma was prunelike with strong vanilla character.

Beans killed by scratching required about 1 day to become brown, which was somewhat longer than in the other treatments. The beans were oily during sweating, but somewhat less after drying. Some flowery aroma developed during drying. Those scratched to 2 cm. from the stem end had a firm lump in this position which later disappeared and left the stem end flexible. Those scratched along the entire length became woody at the stem end. The texture was more flaccid than those killed with hot water but was nevertheless firm. After conditioning, the color was dark brown with some reddish cast and the oiliness had subsided. The aroma was strong, flowery, very agreeable, and fully developed. Of all the treatments, only this one resulted in vanillin crystals being formed on the surface of the beans. It was also noted that the samples were completely cured in about

[4] ARANA F. E. VANILLA CURING AND ITS CHEMISTRY. Puerto Rico (Mayaguez) Agr. Expt. Sta. Bul. 42, 17 pp., illus. 1944.

2 months of conditioning. Those scratched the entire length of the bean had woody stem ends, but those scratched to 2 cm. from the stem end did not.

The combination treatments consisting of a hot-water kill followed by scratching, as well as that consisting of scratching followed by a hot-water kill, resulted in a product similar to that of hot-water kill alone. However, the vanilla aroma was not so strong in the scratched beans, and plugs of resin formed in the scratches which lent a fermented aroma to the beans.

The samples killed by freezing were characterized by a reddish-brown color and a very flaccid texture that persisted throughout the curing process. During sweating the beans were slightly oily, but this had subsided by the time conditioning was over. The product was smooth and of sweet, suave aroma with less vanilla character than those killed by the hot-water or scratching process.

The combination treatments, hot water-freeze, freeze-hot water, scratch-freeze, and freeze-scratch, resulted in a product like that of frozen beans except that the aroma was a little stronger in the double-treated samples. The color was reddish brown and the aroma sweet, similar to beans killed by freezing. Of the combinations the scratch-freeze process resulted in the strongest vanilla aroma.

According to the foregoing observations on aroma the three single-killing processes ranked as follows: First, scratching; second, hot water; third, freezing. The scratching method was best when the scratch was not carried all the way to the stem end; woody stem ends were thus avoided. None of the combination procedures were outstanding improvements over the single methods. However, the freezing method was improved to some extent when it was preceded by a scratching or a hot-water kill.

Extracts were prepared from vanilla beans killed by different methods, and the vanillin analyses on a dry-weight basis gave the following results: Hot-water killing method, 2.80 percent; scratched, 3.30; frozen, 2.86; hot water, scratched, 2.85; scratched, hot water, 2.86; hot water, frozen, 3.00; frozen, hot water, 2.66; scratched, frozen, 2.86; and frozen scratched, 1.93 percent.

Scratching the beans resulted in the highest vanillin content and it was previously noted that the product appeared to be superior. However, organoleptic tests made with ice cream prepared from the extracts indicated that the extract made from beans killed by hot water scored best and those killed by freezing scored second. The difference between the two was not significant. Both were superior by high significance to the extract of beans killed by scratching. The latter was significantly superior to the blank. In appearance, the scratched beans seemed to be superior because of the rapid rate of curing, vanillin crystallization, high vanillin content, and good aroma. However, according to the organoleptic ice cream test, which is a more objective criterion, the beans killed by hot water and freezing were superior. This indicates again that the appearance criteria are not necessarily related to final quality.

EXPERIMENTAL CURING METHODS

WHOLE, CUT, AND GROUND BEANS

In the following work the above criterion of vanilla quality was used in some cases in conjunction with the ordinary tests. The curing of vanilla using whole, cut, and ground beans has been the subject of extensive investigation at this station. A previous report [4] presented the results obtained in experimental curing procedures as well as in biochemical studies of the enzymatic processes involved in curing.[5] These experiments emphasized the importance of oxidative changes that occur during curing. It was found that the principal oxidizing reactions of the vanilla enzyme system were brought about by an oxidase. That this oxidase could also oxidize vanillin and other phenols has been determined. Thus it becomes of interest to study the role of oxygen in the process. The present experiment was designed primarily to show the effects of aeration on the bean tissue during curing. A second objective was to determine whether such a method could be used to simplify the curing methods commonly used.

Duplicate 200-gm. samples of fresh beans, harvested when the blossom end became yellow, were used in four different degrees of fineness: (1) Whole, (2) 1-cm. slices, (3) ground through a food chopper, and (4) ground with a pestle with sand in a mortar. The main difference between treatments (3) and (4) was that, in the former, few cells would be ruptured while in the latter most cells would be broken. The samples were then oven-killed at 60° C. for 24 hours, sweated until the whole beans were flexible, dried to 28.6 percent of the fresh weight, and conditioned in closed jars at room temperature.

The whole beans became dark brown, oily, and developed a suave, flowery vanilla aroma. The beans cut to 1-cm. slices became dark brown and shiny, but showed a green undeveloped character which, during conditioning, turned to a prunelike aroma; vanilla aroma was weak. Samples ground in the food chopper turned brown on top where exposed to air, but became dark brown throughout only after mixing. The aroma was like that of the cut beans except that the prunelike note was even stronger; in fact, it was so strong that no vanilla aroma could be detected. Beans crushed in the mortar also turned brown on top where exposed to air and had to be mixed to obtain a uniformly dark brown mass. While drying, a slight vanilla aroma developed, somewhat like that of the controls but not so strong.

Extracts were prepared from these samples for vanillin analysis and organoleptic tests. It was found that beans cured whole contained 3.45 percent of vanillin, those cut to 1-cm. slices 3.21 percent, those ground in the food chopper 3.65 percent, and those ground with sand 3.79 percent. Apparently the sliced beans lost some vanillin, probably by sublimation, while the ground beans had even more vanillin than the controls because of the intimate contact brought about between the glucovanillin and the hydrolyzing enzyme and because

[4] ARANA, F. E. VANILLA CURING AND ITS CHEMISTRY. Puerto Rico (Mayaguez) Agr. Expt. Sta. Bul. 42, 17 pp., illus. 1944.

[5] See second paper of this series in the Journal of Agricultural Research, vol. 78, No. 11, for further results in enzyme studies.

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of the decreased exposure during which vanillin could sublime. Organoleptic tests made with ice cream showed no differences among the products of these four treatments.

After organoleptic examination of these extracts, Mr. Rosenbaum made the following comments: The extract of the beans cured whole, No. 5, had "a full bodied aroma, a rich Mexican type characteristic well rounded. The best of No. 5 to 10." The extract of the beans cured in slices, No. 6, was "clean and fairly well rounded; more flowery than No. 5, and somewhat lighter in depth and character." The extract of the beans passed through a food chopper, No. 7, was "well rounded with a full-bodied aroma that was close to No. 5 in all characteristics, but seemed to be slightly less full than No. 5." The extract of the beans crushed with sand, No. 8, was "more insipid than Nos. 5, 6, and 7; slightly flowery in character. Lacks well rounded body. Similar to No. 6 generally, but apparently not as well developed." He regarded the products from whole beans and from beans ground in a food chopper as the best.

It is concluded from this experiment that excessive aeration such as that obtained with cut or coarsely ground beans resulted in excess oxidation and a consequent development of a prunelike note in the aroma. Because of the simplicity of curing chopped vanilla these processes appear promising. In spite of the differences in vanillin content, aroma, and color, it is interesting to note that little difference in the flavor of the extracts could be demonstrated.

CURING THE SEPARATE PARTS OF THE FRUIT

Two-hundred-gram samples of beans were sectioned longitudinally to give one sample of the pod wall, or fleshy part of the bean, and another sample consisting of the central seed portion with placental tissue. On curing, the seed and placental tissue turned brown but developed no aromatic character. The pod wall without central seed portion and placental tissue turned brown and developed a strong sweet aroma with some vanilla character during the sweating and drying period. These samples were especially oily inside where the seeds had been removed. The oil became sticky and finally hardened to a resinous film. During conditioning a fermented acid note became noticeable, but vanilla aroma was still present. After conditioning, the vanilla character predominated; however, this character was incomplete as compared to whole cured beans.

Extracts were prepared from these samples for vanillin analysis and organoleptic tests. The seed and placental tissue contained 2 percent vanillin, dry basis, but when the tissue was incubated with emulsin the percentage increased to 2.74, indicating that unhydrolyzed glucovanillin was present. The cured outer wall contained 4.03 percent of vanillin. From the fresh and final weights and the vanillin and moisture contents it was calculated that the outer portion constituted 64.7 percent of the dry matter of the beans and contained 72.8 percent of the vanillin. The remainder in the seed and placental tissue was 35.3 percent of the dry matter and 27.2 percent of vanillin.

Concerning these extracts Mr. Rosenbaum said: The extract of the seed and placental portion has "a very heavy character with a strong

flowery note and a distinct Mexican end aroma. A well rounded extract." The extract of the outer portion has "a fatty odor seemingly somewhat scorched in character. Almost lacking in sweet note but has a heavy Mexican type end product. Not well rounded at all." The extract of whole beans from this same lot was considered to have a "full bodied aroma with a rich Mexican type characteristic; well rounded; best" of samples submitted.

CONDITIONING TEMPERATURES

Previous experimental work in vanilla curing has involved different methods of killing, sweating, and drying but no attention has been given to the final stage, conditioning. It is recognized that considerable change takes place during the initial stages of curing but the principal development of aroma occurs during conditioning. It is therefore of interest to condition the product in different ways to determine whether the final product is affected. One variable that is readily changed is the temperature at which conditioning is carried out. In commercial curing, if warm temperature were desired, conditioning could be done in a loft or a sun-heated shed. If cool temperature were desired, conditioning could be carried out in a basement or a similar cool place.

To determine the effect of temperature during the conditioning stage, two trials were made. In the first trial three lots of 250 gm. each of blossom-end-yellow beans were cured as follows: The beans were frozen solid overnight at −10° C., thawed and dipped in hot water (80°) for three 10-second periods at 30-second intervals. After sweating and drying to 30 percent of the fresh weight, conditioning was carried out in closed cans at three temperatures, 13°, 27°, and 35°. Weekly examinations were made to observe weights, aroma, color, vanillin crystallization, and mold development.

During the curing process no mold developed on any of the samples and no differences in color appeared among the treatments. Within 1 month after the beginning of the conditioning period, the beans at 35° C. had developed a slight aroma. Subsequently the aroma of these warm-conditioned beans developed more fully and had a pungent note characteristic of Mexican vanilla. Those kept at room temperature were characterized by a more flowery aroma typical of Puerto Rican vanilla and eventually the aroma was more developed than was the case with those conditioned at 13°. However, the cold-conditioned beans had a sweet odor early in the experiment before either of the other groups developed a definite aroma.

Vanillin crystals appeared after 6 months' conditioning on the beans kept at low temperature. Those at room temperature had crystals within 10 days, but those conditioned in the oven produced no crystals.

Samples for tasting were taken 6 months after conditioning was started. In all samples the seeds were tasteless and sandy. Those kept at the lowest temperature had the most pleasant aroma but, as the conditioning temperature increased, the aroma was stronger. The outer wall of those kept at 35° C. was bitter and aromatic and had a taste similar to that of chewing tobacco.

After 7½ months of conditioning, moisture determination showed that beans kept at low temperature contained 27.9 percent of moisture, those kept at room temperature 21.9, and those in the oven 15.8 percent. Vanillin analysis by the method used by the Association of Official Agricultural Chemists showed that on the dry basis all the samples contained about 3.5 percent vanillin.

Ice cream samples were made with the extracts from the lots of vanilla beans that had been conditioned at the three different temperatures. The samples from the beans conditioned in the oven had the most superior flavor, those stored in room temperature the second best flavor, and those stored in the refrigerator the least desirable flavor.

In a second trial beans treated according to the following precedure were used. The samples were killed by immersing them three times in hot water at 80° C. for 10 seconds at 30-second intervals. Following this the beans were kept in blankets for 1 day and then oven-sweated in blankets at 45° until flexible. After drying at room temperature to 28.6 percent of the freight weight, the beans were conditioned in sealed cans using four different temperatures: 45°, 35°, 27°, and 13°. After conditioning for 6 months it was found that those conditioned at 45° had the strongest vanilla aroma accompanied by a sweet prunelike note. The beans conditioned at 35° were not so strong but were more suave. Those kept at 27° and 13° were comparatively poor. Apparently high-temperature conditioning brought out background fixative qualities not so noticeable in the ordinary cured material. It is also important to note that the beans at 45° were completely cured after about 3 months of conditioning.

Extracts were prepared from these samples and when they were filtered through paper to remove fine solids it was noted that the higher the temperature at which the vanilla was conditioned the more rapid was the filtration. This indicated that more fine solids were suspended in the extracts of the beans conditioned at lower temperatures.

Mr. Rosenbaum said concerning these samples conditioned at 13° C., "No. 1 has a typically Puerto Rican light, flowery bouquet that is somewhat insipid all the way down. It has only a light flavor body that owes much of its character to vanillin. The deeper tones are almost lacking." Of those conditioned at 27°, he said: "No. 2, not quite as sweet and flowery as No. 1, but possesses more body, and a slightly better rounded character. Both No. 1 and 2 are lighter in body and depth than No. 3 and 4." Of the third sample, conditioned at 35°, he said: "No. 3 has a rich, winey character backed by the lighter fruity and flowery fractions. Much heavier and better rounded than No. 1 and 2, having a different shading which is closer to No. 4 in type. Some Mexican character." And he described those conditioned at 45° as follows: "No 4 slightly leathery odor indicative of higher fat content. Similar in character to No. 3 with rich, sweet body, and yet a note of the Puerto Rican character. Apparently heavier in end bouquet than any of the others, with a rich Mexican character."

Mr. Rosenbaum summed up his conclusions for all four tests as follows: "No. 1 and 2 are inferior to No. 3 and 4, with No. 1 a shade less desirable than No. 2. No. 3 and 4 are closer together in character,

with No. 3 being my first choice as the best of the lot, but a second examination changed my choice to No. 4 as the best rounded because of its heavier end bouquet."

Vanillin contents were 2.07 percent for the beans conditioned at 45° C., 2.21 percent for those at 35°, 2.55 percent for those at 27°, and 2.73 for those at 13°. The corresponding moisture contents were 14.1, 18.3, 20.5, and 18.5. Organoleptic tests of the extracts in ice cream showed that the extract from beans conditioned at 45° was superior by high significance to the others. Likewise, the extract from beans conditioned at 35° was significantly superior to those from beans conditioned at room and at refrigerator temperatures. Of the latter two, the extract from room-temperature-conditioned beans scored higher than that from cold-conditioned beans, but the difference was not significant.

It is concluded that conditioning at 45° C. was best, but that the beans should not be too dry at the beginning of conditioning. If the beans were dried to one-third of their fresh weight and then conditioned in closed containers at 45°, the product would not be so dry and the conditioning process would be complete in about 3 months.

RECOMMENDED CURING PROCEDURE

On the basis of the experimental work of the authors, the following curing procedure for whole beans is recommended:

(1) As soon as possible after harvest, wipe the beans with a damp cloth and kill by immersing them three times for 10 seconds in hot water (80° C.) at 30-second intervals.

(2) Sweat in sun in blankets, or in a closed oven at 45° C. containing a pan of water.

(3) Dry at room temperature to one-third of the original fresh weight.

(4) Tie beans in bundles, wrap the bundles in heavy paper or seal in jars or cans, and condition at 45° C. for 2 to 3 months until the batch has dried to one-fourth of the original fresh weight.

(5) Remove cured product and allow to air for 2 days.

(6) Wrap and pack for shipment.

SUMMARY

The criteria by which cured vanilla is judged were considered. It is concluded that, since no purely objective methods are available, the best procedure is to apply a subjective method, the results of which can be treated statistically to yield a valid measure of goodness. An organoleptic test well suited for this purpose is described. The test is made with ice cream containing the vanilla extract.

The effect of several killing methods on quality of the cured product was determined. The hot-water kill gave the best product, with freezing second, and scratching third. However, the latter had the highest vanillin content.

Beans cured whole, cut, and ground yielded extracts that were not greatly different in flavoring properties. The cut beans had a prune-like aroma but yielded a satisfactory extract. The ground beans were highest in vanillin.

Curing the pod separate from the seed and placental tissue showed that the pod portion accounted for the main part of the flavor of the bean.

Vanilla conditioned at 45° C. was superior to that conditioned at 35° which, in turn, was superior to the product stored at 27° or 13°.

These experiments show that vanilla-curing methods may be modified in two important respects: (1) Beans may be cut or ground before curing, thus simplifying the handling processes, and (2) conditioning may be carried out at 33°–45° C. to yield a superior product.

INACTIVATION AND VACUUM INFILTRATION OF VANILLA ENZYME SYSTEMS [1]

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INTRODUCTION

The term "curing" is commonly used to denote the over-all changes that take place during the processing of many different types of raw material to a finished product ready for the market. The term covers processing of such diverse products as vanilla, tobacco, cheese whisky, and derris root. The actual changes that occur in all of these curing processes may be basically different but they can be classified as: (1) Those involving the loss of water but no chemical transformations; and (2) those involving principally changes in the chemical composition and in which the loss of water is of only minor importance.

The first class, including such processes as the curing of derris root and of ginger tubers, mainly involves drying; any chemical changes that do occur may be deleterious. For example, under poor drying conditions, derris root curing results in browning and loss of rotenone.[3] Similarly, slowly dried ginger becomes discolored, hard, and of poor quality. The second class of curing, in which hydrolytic and oxidative changes are of more importance than the mere loss of water, is exemplified in such items as cheese and vanilla. This class can be further subdivided into two curing processes, in which the chemical changes are brought about principally by: (1) Enzymes, as represented by the hydrolytic cleavage of glucovanillin in vanilla, and (2) chemical transformation without enzymes, such as occurs in whisky curing. Those processes depending upon enzymatic action can be further classified according to the source of the enzyme into: Foreign enzymes such as those furnished by micro-organisms in the curing of cheese; and intracellular enzymes, which are contained in the material to be cured. These differences in curing processes are shown schematically in figure 1.

[1] Received for publication January 3, 1949.

[2] A previous paper, entitled Criteria for Testing Vanilla in Relation to Killing and Curing Methods, Part I of this work, is published in the Journal of Agricultural Research, vol. 78, No. 11.

[3] JONES, M. A., and PAGÁN, C. CHEMICAL INVESTIGATIONS Puerto Rico (Mayaguez) Agr. Expt. Sta. Rpt. 1945, pp. 17–20.

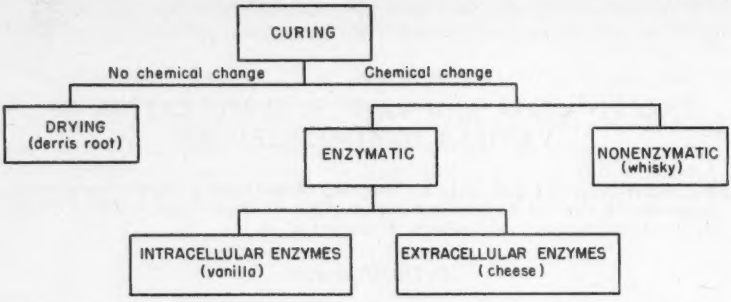

FIGURE 1.—Schematic arrangement to show the differences among curing processes.

THE VANILLA ENZYME SYSTEM

Vanilla curing is a process involving enzymes that are present in the tissue of the beans. An important point of inquiry is the part played by various types of enzymes in the curing process. It is well known that glucovanillin is hydrolyzed by a glucosidase and that there is some peroxidase activity in the beans. It was also found that excessive killing by prolonged immersion in hot water inhibited the browning system of the bean. In an unpublished report, E. K. Nelson, working at this station, stated that expressed juice of vanilla beans did not turn brown if kept from contact with air.

There are, then, two oxidizing enzymes in the whole tissue of the vanilla bean. One of these, a peroxidase, uses hydrogen peroxide. The other, a type of oxidase or aerobic dehydrogenase, utilizes molecular oxygen as acceptor for hydrogen removed from phenols that are being oxidized to stable pigments.

To study the oxidizing enzymes, a modification of the method of Miller and Dawson [4] was used. Briefly the procedure for testing by this technique is as follows: A known amount of enzyme is added to a buffered solution of catechol and a small amount of ascorbic acid in a flask mounted in a water bath. Air is bubbled through the solution and a capillary siphon is used to conduct the solution dropwise into a dish containing excess strong sulfuric acid, pyrogallol, potassium iodide, and starch solution.

The enzyme catalyzes the oxidation of catechol to a quinone which is immediately reduced again by the ascorbic acid. When all of the ascorbic acid is oxidized, free quinone siphons into the receiving dish and oxidizes the iodide ion to iodine which, in turn, yields a blue color with the starch. The acid is present to kill the enzyme coming from the siphon and the pyrogallol to inhibit premature oxidation of the iodide ion by air. The time required for development of the blue color is an inverse function of the activity of the enzyme.

For the work with vanilla beans the crude enzyme was not isolated, but a weighed amount of bean was crushed with sand in a mortar and

[4] MILLER, W. H. and DAWSON, C. R. A NEW METHOD FOR THE MEASUREMENT OF TYOSINASE CATECHOLASE ACTIVITY. Amer. Chem. Soc. Jour. 63: 3375–3382. 1943.

added to the buffered catechol. Since the activity was low no ascorbic acid was added to lengthen the time for the test. The capillary siphon was equipped with a cotton filter to avoid stoppage by tissue fragments. For reaction times in excess of a few minutes the potassium iodide and starch were not mixed with the acid in the receiving dish because, in spite of the pyrogallol, the mixture would take on a purple hue. The siphon was placed above the dish containing the acid, and samples were removed periodically for test with potassium iodide and starch on a spot plate. Pyrogallol was omitted from the reaction. When the reaction time was expected to be long, the siphon was run only at intervals. The technique was tested with excellent results, using potato peel, which is known to contain an oxidase. For the record of results, an arbitrary scale was defined as follows: Positive results in 0 to 3 minutes, high activity; in 3 to 15 minutes, medium activity; in 15 to 30 minutes, low; and over 30 minutes, inactive.

In order to determine whether any ascorbic acid was present in the plant material that would retard the test for oxidizing enzymes, qualitative tests were made by adding methylene blue to a suspension of bean crushed in dilute oxalic acid. No bleaching took place when the blue suspension was exposed to sunlight. The addition of a minute crystal of ascorbic acid to the suspension caused rapid bleaching by sunlight. It can, therefore, be concluded that no appreciable amount of ascorbic acid was present in the bean tissue. The same results were obtained with slices of green and killed beans, but with cured beans the methylene blue was bleached, perhaps by some compound other than ascorbic acid. However, since cured beans gave positive results for oxidizing enzymes, this compound did not appear to interfere with the test.

When green vanilla beans were tested by the above method for oxidase activity, it was found that no color change resulted even after several hours. Of many samples run, only one, otherwise indistinguishable from the others, showed low activity. Sections of large blossom-end-yellow beans showed medium activity in the blossom end, but the middle section of the same bean was inactive. The yellow blossom end of small beans also tested negative.

Crushed green beans killed by immersing in a tube held in water at 65° C. for 3 minutes tested inactive after 1 day. However, beans with yellow and brown blossom ends, killed by freezing, showed high activity after 1 day. With these beans positive results were obtained when the catechol was omitted from the reaction flask, because the enzyme was sufficiently active to act upon the substrate present in the tissue. The tissue of the brown section showed high activity and that of the blossom-end-yellow section, medium activity.

Using bean tissue that showed no activity without the presence of catechol, some tests were made for specificity of the oxidizing enzyme. When tyrosine was substituted for catechol no action resulted. The contents of the flask took on a reddish cast. The addition of tyrosine to a suspension of crushed bean gave a black color after 1 day, indicating that vanilla has some tyrosinase activity. With a mixture of *o*-, *m*-, and *p*-cresol in place of catechol the activity of vanilla enzymes was medium. When these enzymes acted upon added vanillin as the substrate the activity was again medium.

To determine whether molecular oxygen was the acceptor in the reaction, the test was run on deaerated beans. A flask containing several beans was repeatedly evacuated and filled with nitrogen about every 5 minutes for several hours, after which the beans were crushed with sand in a mortar in a box while nitrogen was streamed into the mortar. The bean tissue was transferred to the reaction flask containing the solution previously boiled and then cooled in a nitrogen atmosphere. When the flask had been mounted, nitrogen instead of air was bubbled through and tests were made. After 30 minutes without results 50 mg. of vanillin were added. At the end of an additional 20 minutes without results, air was bubbled through, which produced positive results in 5 minutes. Upon repetition similar results were obtained. It is clear that molecular oxygen is necessary for the main enzymatic formation of quinone bodies in vanilla.

For further characterization of the enzyme the effect of inactivators was studied. It was found that the reaction occurred after 72 minutes in 0.001 M potassium cyanide. It was therefore reversibly inhibited by cyanide. Passing carbon monoxide at the rate of 4 cc. per second with air inhibited the reaction for several hours and developed a dark orange color similar to that brought about by the formation of a carbon monoxide-ferrous cysteine complex. Mercuric acetate, 0.001 M, also inhibited the reaction. When hydrogen sulfide was bubbled in with the air for 50 minutes, the reaction was inhibited; passing air through for 15 minutes after the hydrogen sulfide was shut off resulted in reaction. Whether the hydrogen sulfide reversibly inactivated the enzyme or whether it merely reduced quinones as fast as they were formed is not known.

Copper sulfate and ferric phosphate were tried as activators. Both gave a more rapid reaction but the results with copper were vitiated by the fact that the copper sulfate alone liberated the iodine. The iron salt, on the other hand, did not oxidize the iodide in the receiver but was capable of oxidizing catechol and vanillin in the aerated flask in the absence of plant tissue.

Tests for cytochrome oxidase by the "Nadi" reaction with *p*-diphenylenediamine and alpha-naphthol were positive for green beans both fresh and after killing in hot water and for brown beans killed by freezing. However, the test was negative for the latter type that had stood for 2 weeks.

The presence of catalase in green beans was shown by the decrease of hydrogen peroxide brought about by the addition of crushed beans to a buffered solution of hydrogen peroxide at 0° C., after the method given by Sumner and Somers.[5]

It appears from the foregoing observations that the exidizing enzymes in vanilla form a complex system capable of exidizing a variety of substrates including vanillin itself to quinone bodies and thence to condensed stable pigments. On the basis of the sensitivity to cyanide, the enzyme system appears to contain a heavy metal. Since it requires oxygen as an acceptor and is inactivated by carbon monoxide, the main action of oxidation seems to be carried on by an

[5] SUMNER, J. B., and SOMERS, G. F. CHEMISTRY AND METHODS OF ENZYMES. 365 pp., illus. New York. 1943. (See p. 171.)

oxidase rather than a peroxidase. The manner in which this system should be controlled in order to obtain an optimum cure of vanilla beans remains to be investigated.

EFFECT OF INACTIVATION OF THE ENZYMES

Curing may be defined as indicated in the introduction. However, for the purpose of clarifying thought, it should be stated that many curing processes are not entirely of one type or another. For example, vanilla curing, commonly considered to depend upon enzymes, may, at least in certain phases, be nonenzymatic. It is clear that during the early stages of curing, enzymatic oxidation occurs and it has long been known that the cleavage of glucovanillin to glucose and vanillin is brought about by a hydrolytic enzyme. The importance of enzymes in the later stages of curing was not considered.

With this in mind, therefore, beans were autoclaved at various stages of the curing process to determine the quality of the resulting product. Autoclaving at 120° C. was calculated to destroy the enzyme system completely so that subsequent changes could be considered nonenzymatic in nature. However, it was found that although oxidase, catalase, and peroxidase activities were destroyed by autoclaving, a peroxidase activity later appeared again, at least to a certain extent. For this experiment, triplicate 250-gm. samples of vanilla beans 6 to 8 inches long, with about ½ inch of yellow at the apical end, were killed by immersing three times for 10 seconds at 30-second intervals in hot water (80°). They were then sweated at 45° until flexible and dried at room temperature to 28.6 percent of the fresh weight. The samples were conditioned at room temperature in closed boxes. Groups of samples were autoclaved at the following stages: Immediately after killing, 2 days after killing, at the end of the sweating, and at the beginning of conditioning. At the beginning of the experiment, fresh weights and the moisture content were determined and during the experiment, data on weight, color, aroma, and mold were obtained. At each stage the activities of oxidase, catalase, and peroxidase were measured.

The controls became dark brown and oily after killing. During drying they became more sticky and developed some vanilla aroma. During conditioning the stickiness subsided and the vanilla aroma became more developed. Before killing some catalase activity was found, but after killing none could be shown. Likewise, oxidase, although it is known to be important, was not evident after killing. A high peroxidase activity was noted in the fresh beans. If the value of peroxidase activity of fresh beans is taken as 100 percent, the activity in the controls rose to 115 after killing; 2 days later, 133; dropped to 94 after sweating, and rose to 270 at the beginning of conditioning. After about 2 months' conditioning the activity was 400 percent of that of the fresh beans.

The samples autoclaved after killing stayed green for several weeks and finally became greenish yellow. They were oily, flaccid, and dried the most rapidly of all the samples. During drying the sticky resin became harder and the beans took on a burnt fermented aroma unlike the aroma of vanilla. The same properties were noted during con-

ditioning except that the oiliness subsided and mold developed. No vanilla aroma could be detected. The peroxidase activity dropped to 19 percent and was 20 at the beginning of conditioning.

The beans autoclaved 2 days after killing were light brown, oily, and less flaccid in texture and less disagreeable in aroma than those autoclaved immediately after killing. Upon drying, the resinous oil hardened and some mold developed but was eradicated.[6] After conditioning, the beans were neither oily nor sticky and did not develop a vanilla aroma. The peroxidase activity dropped from 133 to 16 after autoclaving and then recovered to 36 at 12 days, 29 at 43 days, and after 3 months was again 16.

The samples autoclaved 12 days after killing were more like the controls throughout the curing except that the vanilla aroma was not so well developed and the color was lighter brown. The peroxidase activity dropped to 9 percent of the original value upon autoclaving and then recovered to 16 at 43 days, and 12 percent at 3 months.

The beans autoclaved 1 month after killing were similar in quality to the controls; they became dark brown, were not flaccid, and developed a vanilla aroma which was as strong as that of the controls. The peroxidase activity dropped to 23 after autclaving and to 13 after 2 months' conditioning.

Analyses showed that the controls contained 2.44 percent of vanillin, dry basis. Those autoclaved immediately after killing contained 0.31 percent; 2 days after killing, 0.46 percent; 12 days after killing (the end of the sweating process), 1.54 percent; and 42 days after killing (beginning of conditioning), 1.99 percent. The extract of the beans autoclaved immediately after killing was light brown and, as the time of autoclaving after killing was increased, the color was darker. It was concluded that the main part of the hydrolysis of glucovanillin occurred during the first 2 weeks but that some hydrolysis took place during the early part of conditioning. Half of the glucovanillin was hydrolyzed during the first 9 days after killing.

From the results obtained it appears that the principal changes brought about by enzymes occurred during the first 2 weeks of curing. Enzymatic transformations are important during killing and gradually become less so until, by the end of the sweating period, enzymatic changes are unimportant. The fact that peroxidase, after being inactivated by autoclaving, appeared to recover to about the same extent in all of the treatments, indicates that this activity is probably not responsible for changes subsequent to sweating. This is also indicated by the fact that, although the peroxidase activity was exceedingly high in the controls and comparatively low in those autoclaved 12 days and 43 days after killing, the vanilla characteristics were about the same in all cases.

Presumably, then, since the enzymatic changes take place during the first few days of curing and since the principal development of vanilla aroma occurs during the conditioning, a considerable part of the curing process must be nonenzymatic.

It was also found that oxidase, as well as peroxidase, "came back."

[6] "Mycoban" (sodium propionate), either in saturated alcoholic solution or as a powder, has been found to be rather effective in eradicating mold on vanilla.

This was shown by autoclaving two samples of cut beans, after which one was kept sealed while the other was exposed to air but not enough to allow it to dry. After 2 weeks the pieces exposed to air began to brown but those sealed remained green. After 6 months the exposed sample had a weak, sweetish aroma, not like vanilla, while the sealed sample was still green. Therefore, although recovery of oxidase could not be shown by a direct measure of oxidase activity, the foregoing observations showed that an apparent "rejuvenation" occurred.

In the light of recent knowledge concerning nonenzymatic browning (Maillard reaction) the foregoing observations might be otherwise interpreted. The apparent rejuvenation was probably illusory in that the chemical reactions which indicated the activity of the enzymes may have occurred because of the chemical reactivity of products formed during the nonenzymatic browning reactions. Viewed in this manner, it may be said that no reactivation of enzymes occurred and further that, while the hydrolysis of glucovanillin and part of the coloration were due to enzymatic changes, a considerable part of the flavor development was due to nonenzymatic processes. This experiment then suggests a starting point for further study of the relation between flavor development and the browning reaction.

VACUUM INFILTRATION OF ENZYMES

It is well known that an enzymatic step is involved in the hydrolysis of glucovanillin and in some of the oxidative changes that result in browning. An experiment was conducted, therefore, to learn more about the general nature of the enzyme system that acts in the curing process. Cut beans were autoclaved to inactivate the natural enzymes and were then infiltrated with crude enzyme extracts of materials whose enzymatic character is known to some extent. The products were then cured and the results noted.

For this purpose 150–gm. samples of blossom-end-yellow vanilla beans cut to 5–mm. slices were autoclaved for 5 minutes at 120° C. An untreated sample was also prepared which was killed by heating in an oven at 60° for 1 day and then sweated until the pieces lost turgidity and became brown. After drying at room temperature to 28.6 percent of the original weight, the control was conditioned in a closed jar. Immediately after autoclaving and cooling the samples to be treated were covered with an extract of the enzyme to be infiltrated and placed in a vacuum chamber. Upon evacuation to 4 cm. pressure, the air in the cells of the plant tissue bubbled out. The vacuum was released about 5 minutes after bubbling ceased and, as air entered the chamber, the cells filled up with the extract in which the pieces were submerged. After a half hour the infiltrated pieces were drained and cured in the same manner as the control.

The enzyme extracts were prepared as follows: One-half percent yeast extract was made by mixing brewer's yeast with water in a Waring Blendor for 10 minutes. The suspension was then centrifuged for 10 minutes, the precipitate rejected, and the clear liquid containing the enzyme was used. A ½-percent solution of emulsin was prepared in the same manner.

To make the oxidase solution 1 pound of frozen mushroom, *Agaricus campestris*, was passed through a food chopper into 2 volumes of cold acetone chilled with dry ice to precipitate the enzyme and wash out the natural substrate and coloring matter.[7] It was filtered immediately and the pulp frozen for 2 hours with dry ice after which it was broken up with 600 cc. of water containing a drop of concentrated ammonium hydroxide. The filtrate from this contained the enzyme.

Vanilla enzyme was prepared in the same manner, using vanilla beans instead of mushroom.

The peroxidase extract was prepared by grinding 1 pound of frozen turnips several times through a food chopper and then squeezing the juice through cheesecloth. One gram of purified diatomaceous earth was added for each 100 cc. of juice and the suspension filtered after 10 minutes through paper on a Buchner funnel containing a layer of filter aid. The liquid which contained the enzyme was clear yellow.

The enzyme activities of the preparation are given in table 1.

TABLE 1.—*Activities of enzyme preparations used for vacuum infiltration of vanilla*

Source	Peroxidase	Oxidase
Vanilla	Medium	High.
Yeast	None	None.
Emulsin	do	Do.
Mushroom	Low	High.
Turnip	High	None.

The vanilla samples were infiltrated with extracts representing a like amount of the other plant materials. This experiment was carried out twice because, in the first run, oxidation took place in the autoclaved samples and considerable mold appeared. However, the results were similar in both trials. The results are summarized in table 2.

It is clear from the results that, after the natural vanilla enzymes were inactivated, infiltration with an oxidase preparation gave a product that resembled the ordinary cured vanilla more closely than did the products obtained by using other enzyme preparations. In samples treated with oxidase diluted with other enzymes the product was poorer. This experiment further confirms the conclusion that an oxidase is responsible for most of the enzymatic oxidizing action in the development of vanilla aroma that occurs during curing.

SUMMARY

The nature of the process of vanilla curing is discussed.

The oxidase system in vanilla was briefly investigated.

The activities of the oxidase, peroxidase, and catalase during curing were measured and it was concluded that the main enzymatic change is due to an oxidase system.

[7] MILLER, W. H., MALLETTE, M. F., LLOYD, J. R., and DAWSON, C. R. A NEW METHOD FOR THE MEASUREMENT OF TYROSINASE CATECHOLASE ACTIVITY. II.—CATECHOLASE ACTIVITY BASED ON THE INITIAL REACTION VELOCITY. Amer. Chem. Soc. Jour. 66: 514–519. 1944.

TABLE 2.—*Results of vacuum infiltration of autoclaved vanilla beans with some crude enzyme extracts of other materials*

Enzyme		Result		
Source	Nature	Color	Aroma	Remarks
Control		Dark brown	Prunelike vanilla	Good vanilla.
Control [1]		Greenish brown	Weak acid	No cure.
Vanilla	Glucosidase, oxidase, peroxidase.	Dark brown and dark green.	Weak acid, some sweet note, no vanilla.	Moldy.
Water		Greenish brown	Weak acid	No cure.
Yeast	Alpha-glucosidase	Dark brown	Acid, no vanilla	Moldy.
Emulsin	Beta-glucosidase	Dark brown outside and yellow inside.	Acid, no vanilla	Poor.
Mushroom	Oxidase	Dark brown	Prunelike vanilla	Weaker than control.
Turnip	Peroxidase	Dark brown	Acid	Poor.
Mixture	Oxidase-peroxidase	Light to dark brown	Acid, slight vanilla	Not so good as control.
Mixture	Emulsin-oxidase	Light to dark brown	Prunelike vanilla	Weaker than control.
Mixture	Emulsin-peroxidase	Dark brown	Acid	Poor.
Mixture	Emulsin-peroxidase-oxidase.	Light to dark brown	Prunelike, slightly acid.	Not so good as control.
Mixture	Yeast-emulsin	Light to dark brown	Acid, slightly sweet, no vanilla.	Poor.
Mixture	Yeast-oxidase	Light to dark brown	Prunelike vanilla	Weaker than control.
Mixture	Yeast-peroxidase	Light to dark brown	Acid	Poor.
Mixture	Yeast-oxidase-peroxidase.	Light to dark brown	Acid	Poor.
Mixture	Yeast, emulsin, oxidase, peroxidase.	Light to dark brown	Acid	Poor.

[1] Autoclaved but not infiltrated.

It was found that the main enzymatic changes occur during the first 12 days of curing but the main development of flavor occurred after this time. A considerable part of the curing process appears to be nonenzymatic.

Vanilla with inactivated enzyme system was infiltrated with enzyme extracts of several types to find that an oxidase-type extract gave a product most closely resembling vanilla.

QUALITY OF CURED VANILLA IN RELATION TO SOME NATURAL FACTORS [1]

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INTRODUCTION

During the vanilla-curing season of 1945–46 studies were made for the purpose of correlating the quality of cured vanilla with several natural factors which, by their effects on the developing bean, might affect the quality of the cured product. For this work a standard curing and testing procedure was used so that the quality of the cured beans would depend only on the conditions under which they were grown and harvested.

VARIATION OF QUALITY DURING SEASON

The fruit of *Vanilla fragrans* (Salisb.) Ames reaches its full size several months before it is ready for harvest. The time required for the full-sized pod to ripen for harvest varies to the extent that in Puerto Rico some pods are ready for harvest early in November, most are ready in late December and early January, while some are not ripe until February. The time at which the peak of the harvest season occurs varies somewhat from year to year, depending on climatic conditions. Also it is notable that the early beans are usually smaller than those that ripen later. Since it was considered probable that the time of the season at which the pods ripen might affect the quality of the cured product, some trials were made in which beans were harvested early in the season, at the peak of the season, and in late season. Beans were collected on November 3, December 27, and January 31. To insure a uniform degree of maturity, all beans were harvested when the blossom end was yellow. The location of the vanillery was near Mayaguez on Las Mesas at an elevation of 600 feet.

Soon after harvest the pods were wiped with a damp cloth and killed by immersing in hot water (80° C.) three times for 10 seconds at 30-second intervals. This was followed by sweating in an electric oven at 45° until the beans were flexible. They were then dried on drying racks at room temperature to one-third of fresh weight. Conditioning was then carried out by storing in closed vessels at 45° for 2 months by which time the weight was one-fourth of the fresh weight. No mold developed on any of the samples.

[1] Received for publication January 3, 1949.

[2] "Criteria for Testing Vanilla in Relation to Killing and Curing Methods," and "Inactivation and Vacuum Infiltration of Vanilla Enzyme Systems," are parts of this same series and are published in the Journal of Agricultural Research, vol. 78, no. 11.

The beans harvested early in the season were 4½ to 7½ inches long with most of them about 5½. The moisture content was 80.07 percent. During sweating and drying a strong vanilla aroma developed which was considered very good. After the beans had been conditioned the aroma was even stronger and the beans were somewhat oily and black. In general, the cured product resembled Mexican vanilla. No vanillin crystallized on the surface of the beans.

The beans harvested at the height of the season were 8 to 9½ inches long and averaged 74.48 percent moisture. During sweating and drying the product developed a slight vanilla aroma accompanied by a sweetish note. The aroma lacked strength and was definitely inferior in quality to that of beans harvested early in the season. During conditioning the aroma improved but still lacked strength. The beans were shiny and oily which gave them a pleasing appearance although they were somewhat light in color. No vanillin crystallized on the beans.

The beans harvested late in the season were 8 to 10 inches long and averaged 79.66 percent moisture. During the sweating and drying the samples developed a bouquet similar to that of the beans harvested at midseason. The aroma improved during conditioning. A considerable amount of vanillin crystallized on the surface of the beans, which improved the appearance of the product, but otherwise there was little difference in aroma and appearance of these beans and those harvested at midseason.

The moisture and vanillin contents of the cured products are tabulated in table 1.

TABLE 1.—*Moisture and vanillin contents of cured vanilla harvested at different times of the harvesting season*

Harvesttime	Moisture	Vanillin [1] [2]
	Percent	*Percent*
Early, Nov. 3	21.37	2.67
Middle, Dec. 27	27.61	3.59
Late, Jan. 31	26.82	4.17

[1] The vanillin content was calculated on the dry basis.

[2] Vanillin analyses were made according to the A. O. A. C. method. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS. . . . Ed. 5, 757 pp., illus. Washington, D. C. 1940. (Pp. 320-321.)

Extracts representing equal amounts of dry vanilla were prepared [3] and compared by a method recommended by Gnadinger.[4] This method consisted of diluting the extract with 19 parts of water to 1 part of extract. According to this test beans harvested early in the season had a stronger vanilla flavor than beans harvested later in the season. Concoctions containing 1 cc. of extract in 50 cc. of milk were also tested with the same results.

[3] The method used for the preparation of extracts was outlined in the first paper of this series, published in the Journal of Agricultural Research, vol. 78, no. 11.

[4] GNADINGER, C. B. VANILLA. 60 pp. Minneapolis, Minn. 1929.

From these results it appears that beans harvested early in the season, although smaller and lower in vanillin content, cure to a superior product as judged by aroma and flavor.

EFFECT OF MATURITY OF VANILLA ON QUALITY OF CURED PRODUCT

In previous work at this station it was shown that beans harvested when the blossom end had turned yellow gave a cured product superior in aroma and in vanillin content to that from beans harvested entirely green. In other work beans harvested after they had turned brown on the vine were shown to give a cured product higher in vanillin than the product from green beans but lower than that from blossom-end-yellow beans. Because of these observations it is recommended that vanilla be harvested when the blossom end is light yellow and before splitting begins. Split beans are discounted on the market although some claim that the split product yields a superior extract. The work was repeated with beans picked at several stages of maturity: (1) Entirely green, (2) blossom end yellow, (3) blossom end brown, and (4) entirely chocolate. The beans used were grown in a vanillery on the station grounds.

During sweating the beans harvested green developed a slightly fermented aroma which disapeared during drying. Vanilla aroma was faint but improved during conditioning and was acompanied by a sweetish note. However, the vanilla aroma did not become so strong or as true as that of a high-quality cure. The beans were dull light brown with a gummy texture. It was clear that vanilla should not be harvested at this stage of maturity.

The samples harvested with the blossom end yellow gave a good cured product, dark brown, oily, and with a pleasing vanilla bouquet.

Those harvested with the blossom end brown developed a fruity aroma which was agreeable but lacking in true vanilla character. Later the aroma became fragrant and similar to that of beans killed by scratching. Most of the beans were split. Much vanillin crystallized on the surface of these beans.

Beans harvested when brown cured to a product with an aroma similar to but weaker than that of the beans harvested with the blossom end brown. There was profuse vanillin crystallization on these beans.

Extracts were prepared and vanillin analyses made. The data are given in table 2.

TABLE 2.—*Analyses of vanilla beans harvested at several stages of maturity*

Stage of maturity when harvested	Moisture content		Vanillin, dry basis
	Fresh	Cured	
	Percent	*Percent*	*Percent*
Green	82.14	33.92	2.86
Blossom end yellow	80.47	24.12	3.50
Blossom end brown	67.05	22.76	3.79
Brown	59.07	12.62	3.40

From the figures presented in the table it appears that the best vanilla is obtained from beans harvested when the blossom end is yellow. This corroborates data obtained in previous experiments.

ALTITUDE OF PLANTING AS A FACTOR IN QUALITY OF CURED VANILLA

Among the many possible factors that might affect the quality of the cured product is the altitude at which the vanillery is located. Temperature apparently has something to do with the time of bean set; hence, it is possible that an altitude effect due to temperature difference could determine to some extent the quality of cured beans. In a preliminary effort to measure the effect of altitude on the quality of cured vanilla, an experiment was made in which vanilla beans were harvested from vanilleries at several altitudes. No final evaluation is possible at this time because in this experiment there were several other differences among the samples beside those due to altitude alone.

Beans were harvested when the blossom end was yellow and cured by the process outlined previously. Samples were obtained from the following locations and altitudes: Mayaguez, 80 feet altitude; Las Mesas, 600 feet; Morovis, 700 feet; and Castañer, 1,800 feet.

During the sweating phase of the curing, beans from Mayaguez developed a strong vanilla aroma which persisted throughout the process. The product was oily, shiny, dark in color, and possessed a very good aroma and appearance.

The samples from Las Mesas, although they appeared to be the best beans when fresh, did not develop a full vanilla aroma upon curing. The character of the aroma was faint and had a sweetish note. The beans were somewhat oily and lighter in final color than those from Mayaguez.

Beans harvested at Morovis developed an appearance and aroma characteristic of beans harvested entirely green. They were dull, light brown in color, not oily, and gummy in texture. Only a slight vanilla aroma developed.

The Castañer product developed a suave vanilla aroma with a sweetish note during the sweating. There was also a foreign, somewhat flowery odor present. After conditioning, the beans had a good vanilla aroma which was not so strong as that of the beans from Mayaguez. No vanillin crystallized on the surface of any of the samples. The data on beans from different altitudes are presented in table 3.

TABLE 3.—*Analyses of vanilla beans harvested at several altitudes in Puerto Rico*

Location	Elevation	Moisture content		Vanillin content, dry basis
		Fresh	Cured	
	Feet	*Percent*	*Percent*	*Percent*
Mayaguez	80	77.07	25.50	3.79
Las Mesas	600	81.26	36.06	3.45
Morovis	700	81.58	21.97	2.96
Castañer	1,800	78.82	30.85	3.75

Extracts prepared from these beams were tested in milk and in water and ranked as follows in decreasing order of quality: Mayaguez, Castañer, Las Mesas, Morovis.

From this experiment it appears that any effect that the altitude at which the plant grows may have on vanilla quality was masked by other factors such as soil, mulch, or rainfall distribution.

QUALITY OF VANILLA FROM DISEASED PLANTS

Another factor that might be expected to determine, at least to some extent, the quality of vanilla beans is the state of health of the plant. It is known that the quality of many fruits is influenced by the vigor of the plant. Although vanilla root rot is a localized disease, it causes general wilting of the plant and ultimate death. Therefore, it was considered possible that beans from plants with root rot might differ in the quality of the cured product from those grown on healthy plants.

To test this hypothesis duplicate samples of beans were collected from 16 plants, 8 of which were healthy and 8 of which were suffering from root rot. The beans from healthy plants were 7½ to 9 inches long. The samples cured by the procedure outlined in the first section appeared to be similar, and no differences in aromatic qualities could be detected. Both lots had good vanilla character. There was no vanillin crystallization or mold development on any of the samples. The analytical data are given in table 4.

TABLE 4.—*Analyses of vanilla beans from healthy and from diseased plants*

State of health of plant	Moisture content		Vanillin content, dry basis
	Fresh	Cured	
	Percent	*Percent*	*Percent*
Healthy	78. 64	33. 38	3 45
Diseased	80. 22	36. 56	2. 97

Extracts of these samples were tested organoleptically in water and in milk with the result that no differences in flavoring properties could be established. From these results it can be concluded that, although the effect of vanilla root rot is to decrease the quantity of beans by weakening the plant, the disease does not affect the quality of the cured product.

SUMMARY

Some experiments were made for the purpose of relating the quality of cured vanilla with the conditions under which the plant was grown.

It was found that vanilla beans that ripened early in the harvesting season, although smaller than those that ripened later, gave a cured product of somewhat higher quality than those harvested at midseason or late in the season.

Beans were harvested at several stages of maturity and it was found that green beans cured to an inferior product. The best cured vanilla was obtained from beans harvested when the blossom end of the fruit was yellow.

As far as could be determined, the altitude of the vanillery at which the beans were harvested did not have any effect on the quality of the cured beans. If any differences due solely to altitude were present, they were masked by other factors.

Beans from diseased plants were about the same in final quality as those from healthy plants, but those from diseased plants were somewhat smaller.

RATE OF DEVELOPMENT OF CALIFORNIA RED SCALES RESISTANT AND NONRESISTANT TO HYDROCYANIC ACID GAS, AS AFFECTED BY TEMPERATURE [1]

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INTRODUCTION

The rate of development of the California red scale (*Aonidiella aurantii* (Mask.)) in California has been discussed by Quayle [3] and by Bliss, Broadbent, and Watson.[4] Their work was done before the genetic basis of differences in resistance to hydrocyanic acid gas had been established by Dickson [5] and Yust et al.[6] In previous work the writer [7] found no differences in reproduction and mortality between resistant and nonresistant scales. Studies on the rate of development of the same two strains were then made to determine whether there were any differences that might affect the relative growth rates of populations of the two strains in the field.

METHODS

The methods of handling and rearing the insects were similar to those described by Munger.[7] The periods measured were the first instar of both males and females and the second instar and the third stage of the females. The third stage included the period from completion of the second molt to production of the first young. The development of the males was not followed after they became recognizable as such in the second instar.

[1] Received for publication May 10, 1948.

[2] The author is indebted to A. W. Cressman, for advice and criticism.

[3] Quayle, H. J. The red or orange scale. Calif. Agr. Expt. Sta. Bul. 222, pp. 99–150, illus. 1911.

[4] Bliss, C. I., Broadbent, B. M., and Watson, S. A. The life history of the California red scale, Chrysomphalus aurantii Maskell: progress report. Jour. Econ. Ent. 24: 1223–1229, illus. 1931.

[5] Dickson, R. C. Inheritance of resistance to hydrocyanic acid fumigation in the California red scale. Hilgardia 13: 515–521, illus. 1941.

[6] Yust, H. R., Nelson, H. D., and Busbey, R. L. Comparative susceptibility of two strains of California red scale to HCN, with special reference to the inheritance of resistance. Jour. Econ. Ent. 36: 744–749, illus. 1943.

[7] Munger, F. Reproduction and mortality of California red scales resistant and nonresistant to hydrocyanic acid gas, as affected by temperature. Jour. Agr. Res. 76: 153–163, illus. 1948.

EXPERIMENTAL RESULTS

DEVELOPMENT AT CONSTANT TEMPERATURES

In the first experiment scales were subjected to a series of five constant temperatures—93.2°, 86.0°, 78.8°, 71.6, and 64.4° F. The extremes of temperature represented in this series were near the upper and lower limits at which the life cycle could be completed. Because of the slow rate of development at 64.4°, observations for all stages at that temperature were made at 24-hour intervals. Observations at the other temperatures were made at 12-hour intervals, except for the third stage, which were also made at 24-hour intervals.

Six lemons were used for tests at each temperature. All the lemons were infested by the half-lemon technique at 78.8° F. over periods ranging from 60 to 90 minutes. Immediately after being infested they were placed in constant-temperature cabinets. On three lemons of each group the resistant strain was on the upper half, and on the other three it was on the lower half. The mid-infesting time was used as the theoretical beginning of the test. During the first instar 60 scales on each half lemon were individually numbered with India ink. Inspections were started before any scales began to transform and were continued until the scales had either died or reproduced, or until the lemon had become hard or spoiled. Except for the time required to examine the scales, and for some minor irregularities in the control apparatus, the temperatures were held within $\pm 0.9°$. Continuous air circulation was provided by blowers or fans. All inspections were made at 78.8°, except for the scales subjected to 64.4°, which were examined at 59°–60.8°. Humidity was maintained in the chambers at approximately 68 percent for 64.4°, 56 percent for 71.6°, 60 percent for 78.8°, 44 percent for 86.0°, and 43 percent for 93.2°.

During the inspections the lemons were removed from the cabinet one at a time. The examination of the scales on each lemon required approximately 7.5 minutes. Radiant heat from the microscope lamp during the examination was nearly eliminated by passing the light through a glass vessel containing copper sulfate in water. The color and appearance of the scale cover was used as the criterion of development. In preliminary work it was found that the end of the first instar of both sexes is marked by a rather definite change in the appearance of the scale cover. The yellow color becomes more orange, and the appearance suggests dryness and opacity. This change is associated with the separation of the scale body from the cover. The end of the second instar in the female is marked by a similar change in appearance, but the scale is much larger and the outline of the body can usually be seen to have drawn away from the cover. The inspections were made under a magnification of 20.7.

The appearance of crawlers under the cover was used as an index of reproduction. The crawlers were usually, but not always, visible through the cover.

To check the uniformity of results in different generations, and to provide data at additional temperatures, a second constant-temperature experiment was carried out with a later generation of scales. Two new temperatures, 82.4° and 75.2° F., were used, and 78.8° was

repeated. The technique was similar to that used in the first experiment, except that inspections were made at 24-hour intervals and the third stage was not measured. Inspections were made at temperatures approximating those in the cabinet. The results of these experiments are summarized in table 1. The values shown are averages for all the insects.

TABLE 1.—*Hours required by red scales for completion of life stages at different constant temperatures*

FIRST EXPERIMENT

Temperature (°F.)	First instar				Second-instar female		Third-stage female		Complete development of females	
	Male		Female							
	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant
93.2	176	189	182	179	278	262	569	550	1,006	960
86.0	186	181	194	189	251	246	444	441	885	873
78.8	220	221	239	228	269	270	495	491	1,002	989
71.6	344	349	361	360	443	449	831	838	1,614	1,621
64.4	604	610	622	631	787	790	1,497	.....	2,837	.....

SECOND EXPERIMENT

82.4	198	198	206	203	250	253	.....	.....	.....	.....
78.8	233	235	249	237	293	270	.....	.....	.....	.....
75.2	277	286	298	301	352	349	.....	.....	.....	.....

The data of the first experiment indicate that the nonresistant scales may grow a little more rapidly than the resistant scales at the higher temperatures and possibly slightly more slowly at the lower temperatures. However, the test at 86° F. was the only one showing a statistically significant difference ($P=0.01$) between the two strains in the time required to complete development. All the differences were very small.

The analysis of variance of the data for 78.8° F. (the only temperature common to the two experiments) showed a significant difference at the 1-percent level between the two experiments. At that temperature scales in the second experiment required from 9 to 14 hours longer to complete the first instar than did those in the first experiment. The resistant female scales required 24 more hours to complete the second instar in the second experiment, but there was no difference in the nonresistant strain. There was also a significant difference between the sexes in the first instar; except for the nonresistant scales at 93.2°, the first instar in the males was consistently shorter than in the females in both experiments.

Except where otherwise stated, 360 scales of each strain were tested at each temperature in each experiment. The fate of all the scales in the two experiments is shown in table 2.

TABLE 2.—*Number of red scales that attained various stages of development in constant-temperature experiments*

FIRST EXPERIMENT

Temperature (° F.)	Died in first instar		Died in second instar		Males in second instar		Died in third stage		Reproduced	
	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant
64.4	37	31	184	178	45	53	82	98	12	0
71.6	83	67	60	56	78	95	70	68	69	74
78.8 [1]	121	99	10	6	113	118	8	6	108	129
86.0	34	32	27	17	126	148	27	16	146	147
93.2	39	15	52	35	95	123	153	135	21	52

SECOND EXPERIMENT

75.2	50	70	85	73	99	103				
78.8	56	40	50	49	126	145				
82.4	42	50	55	55	117	95				

[1] Only 358 nonresistant scales were used at this temperature.

In the first experiment the mortality of the first-instar scales appeared rather high, but it was followed by a low mortality in the second instar. Between 64.4° and 86° F. the number of females that survived to reproduce increased with increasing temperatures. More of the resistant females produced young at 64.4° and more of the nonresistant reproduced at 93.2°; otherwise there was little difference in survival of the two strains. In the second experiment no large differences in mortality occurred between the strains. At 78.8° fewer scales died in the first instar than in the first experiment, but there was a correspondingly higher mortality in the second instar.

DEVELOPMENT AT FLUCTUATING TEMPERATURES

Two experiments were made to determine the relative rate of development of the two strains of the California red scale under fluctuating temperatures. The first experiment was designed to represent the general range of moderate spring and summer temperatures. The scales were exposed to temperatures that fluctuated uniformly between a minimum of 64.4° F. at 5 a. m. and a maximum of 86° at 5 p. m. each day. Inspections were made at 12-hour intervals for the first and second instars and at 24-hour intervals for the third-stage female.

The temperatures for the second experiment, representative of high summer temperatures which may occur in interior California, were selected from the thermograph records of Corona over a 7-week period from July 8 to August 26, 1935. The records for this period were transcribed to the reproducing element of a variable thermostat in order to recreate the same conditions in the laboratory. In this period 35 days had a minimum of less than 60° F. In 11 days the minima ranged from 47.5° to 50°. Twenty days had a maximum of more than 100°, and on 7 days the maximum temperature ranged from 105.1° to 107.8°. Inspections were made at 24-hour intervals. The second molt of the scales was completed, but no reproduction

occurred within the 7-week period. The first 4 weeks of the program were then repeated, after which the lemons were transferred to a constant temperature of 77° for the last 5 days of the experiment. During the developmental period of the scales the lemons were in good condition, but near the end of the experiment they were beginning to harden. In each program 360 scales of each strain were tested.

The results of these experiments are shown in table 3.

TABLE 3.—*Hours required for development of red scales in 2 fluctuating-temperature programs*

Stage and sex	64.4° to 86° F.		Corona high-temperature program	
	Resistant	Nonresistant	Resistant	Nonresistant
First-instar male	299.6	296.5	369.5	354.8
First-instar female	319.1	303.0	366.8	360.7
Second-instar female	361.6	349.3	455.4	456.6
Third-stage female	694.4	676.9	767.8	758.1
Complete development of females	1,366.0	1,321.1	1,588.5	1,570.7

In the experiment at moderate temperatures the resistant scales required about 45 hours more than the nonresistant scales to complete their development, a difference that was possibly significant ($P=0.05$). In the other experiment with a wider range of temperatures the difference between strains was not significant.

The fate of the scales in the experiments at fluctuating temperatures is shown in table 4.

TABLE 4.—*Number of scales that attained various stages of development in 2 fluctuating-temperature programs*

Fate of scales	64.4° to 86° F.		Corona high-temperature program	
	Resistant	Nonresistant	Resistant	Nonresistant
Died in first instar	55	63	39	37
Died in second instar	85	64	35	30
Distinguishable as males in second instar	97	114	115	122
Died in third stage without reproducing	22	14	21	24
Reproduced	101	105	150	147

There was no apparent trend in the mortality of the scales in the two experiments. The numbers that reproduced were nearly equal.

DISCUSSION

In these experiments the differences between strains, if any, were very small and were not conclusively demonstrated. The largest difference observed was about 3 percent of the total period. Such differences, if occurring consistently, would require a long period of time to have much biological effect on the population and seem unimportant relative to the selective action of hydrocyanic acid gas that has been demonstrated in fumigation of mixed populations.

The records on mortality in these experiments, as well as the more extensive data reported earlier,[8] have not shown any consistent differences between strains.

SUMMARY

The rate of development of strains of the California red scale (*Aonidiella aurantii* (Mask.)) resistant and nonresistant to hydrocyanic acid gas has been studied under constant temperatures ranging from 64.4° to 93.2° F. and under two fluctuating-temperature programs. One fluctuating-temperature program, representing moderate spring and summer conditions, ranged from 64.4° to 86°; the other, representing hot interior California conditions, ranged from 47.5° to 107.8°. In two of the tests (64.4° to 86° fluctuating and 86° constant) small differences were detected between the strains. There were no statistically significant differences at other temperatures, but there was a suggestion that a slight difference may exist between the strains. The resistant scales may develop less rapidly than the nonresistant scales at high temperatures and perhaps more rapidly than the latter at low temperatures.

In the constant-temperature experiment a small but significant difference in the rate of development of scales of two generations was found.

In the range of temperature from 64.4° to 93.2° F. the number of females that survived to reproduce increased with increasing temperatures. A larger number of resistant than nonresistant females produced young at 64.4° and larger number of the nonresistant females reproduced at 93.2°.

In the fluctuating-temperature experiments there was no apparent difference in mortality between the strains of scales. In the two experiments the numbers of scales that lived to reproduce were nearly equal.

[8] See footnote 7, p. 451.

GROWTH OF FIRST-GENERATION CROSSBRED DAIRY CALVES[1]

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INTRODUCTION

One of the chief questions of interest in all cross-breeding investigations is whether or not the crossbred progeny will exhibit any evidence of heterosis, or so-called "hybrid vigor." In view of the fact that heterosis, as indicated by the increased rate of growth of the progeny, has been obtained by crossing breeds in some other kinds of livestock, it might reasonably be expected that crossing the dairy breeds would produce similar results. This paper presents an analysis of the rate of growth of first-generation crossbred dairy calves as compared with the growth standards for purebreds.

EXPERIMENTAL PROCEDURES

A cross-breeding project with dairy cattle was set up early in 1939 by the Bureau of Dairy Industry at the Agricultural Research Center, Beltsville, Md. The breeds involved are Holstein, Jersey, Guernsey, and Red Dane. Red Dane cattle rank next to Holsteins in size, mature cows weighing from 1,300 to 1,350 pounds. They are shorter legged, somewhat blockier, and have a tendency to be proportionately longer-bodied than the other dairy breeds.

The following crosses have been made: Holstein × Jersey, Jersey × Holstein, Holstein × Guernsey, Holstein × Red Dane, Red Dane × Holstein, Jersey × Red Dane, Red Dane × Jersey, and Red Dane × Guernsey. The first breed mentioned in each cross is that of the sire, the second that of the dam. Early in the experiment a few Jersey × Guernsey crosses were made, but the crossbred progeny are too small in number to be considered in this analysis. Only in the Holstein × Jersey combinations are the numbers large enough for separate studies of the reciprocals. Unless otherwise stated the data are for female calves only.

All crossbred calves in this study were raised by the same procedure as is used in raising calves in the breeding herds at Beltsville, which is as follows:

[1] Received for publication June 28, 1948.

The feeding and management of the calves conform to good dairy practice. The calves are separated from the dams shortly after birth and are hand-fed in small pens. Whole milk is fed for the first 4 to 8 weeks, depending on the vigor of the calf, after which skim milk is substituted. Skim-milk feeding is discontinued after 6 months of age. Starting at about 3 weeks of age, the calves are fed alfalfa hay and a small amount of grain. The grain mixture consists of corn, oats, wheat bran, and linseed meal. The grain ration is gradually increased to a maximum of 3 pounds daily by the time skim-milk feeding is discontinued. After 12 months of age the heifers are housed in a stanchion barn and, in addition to hay and grain, they are fed a small ration of corn silage in the winter and are pastured during the summer months.

Every effort is made to insure the health of the calves. They are kept under close observation at all times, and any ailment is given prompt veterinary treatment.

Few of the male calves are retained in the experimental herds; hence only their birth weights are available for consideration here. Monthly weights for the heifer calves are taken from birth, but the weights after 18 months will not be considered here because the heifers are bred first at about 15 months of age and the effect of pregnancy becomes too much of a disturbing influence after 18 months.

The measures of growth used in this analysis are the live weight and a set of five skeletal measurements as follows: Height at withers, width of fore chest, depth of fore chest, width of hips, and the total length from the withers to the pin bones. Live weight is an average of the weight on 3 consecutive days, taken at intervals of 30 days, up to 12 months of age. Thereafter, all heifers are weighed on the first day of each month, this weight being used as the weight of the animal for the age unit nearest to the dates on which the weighings were made. The skeletal measurements are taken at 6, 12, and 18 months of age.

For a number of years, these same measures of growth have been applied to the calves in the purebred Holstein, Jersey, and Red Dane herds at Beltsville. From the resulting data, standards of weights and measurements have been calculated for use in making comparisons between the purebred calves and the crossbreds of these three breeds.

A study of growth data from the Beltsville herds and of similar data gathered at other institutions makes it seem unsound to compare such data from one herd against growth standards calculated from other herds where conditions of feeding and management are somewhat different. For this reason, the only standards of growth used in this analysis are those developed from Beltsville data.

Table 1 shows the standards of live weight for purebred Holstein, Jersey, and Red Dane calves; and table 2 shows the standards of skeletal growth for these three breeds.

TABLE 1.—*Standards of live weight for purebred dairy calves*

Age (months)	Holsteins			Jerseys			Red Danes		
	Animals	Average weight	Coefficient of variability	Animals	Average weight	Coefficient of variability	Animals	Average weight	Coefficient of variability
	Number	*Pounds*	*Percent*	*Number*	*Pounds*	*Percent*	*Number*	*Pounds*	*Percent*
Birth	229	95.8±0.79	12.53	254	55.2±0.46	13.28	49	80.8± .1.56	13.53
1	224	118.6± .93	11.72	240	71.7± .60	12.91	48	106.6± 2.16	14.02
2	223	159.4±1.42	13.31	235	100.3±1.16	17.67	49	144.8± 3.14	15.18
3	223	211.1±1.58	11.15	229	136.9±1.21	13.38	49	192.7± 3.71	13.47
4	222	268.2±1.95	10.84	230	178.6±1.49	12.65	49	244.7± 4.28	12.24
5	221	330.7±2.24	10.05	229	226.3±1.73	11.58	48	298.6± 5.12	11.87
6	218	390.9±2.67	10.08	229	275.1±1.94	10.65	48	350.3± 5.46	10.80
7	217	449.1±2.90	9.50	224	323.2±2.26	10.49	48	397.6± 5.73	9.98
8	216	502.7±3.39	9.90	221	365.2±2.47	10.06	48	437.4± 5.42	8.58
9	215	553.6±3.75	9.92	219	404.2±2.67	9.78	48	479.5± 5.67	8.20
10	211	605.0±3.88	9.31	217	441.0±2.93	9.80	48	520.9± 6.09	8.10
11	210	654.9±4.08	9.03	215	477.4±2.88	8.84	48	559.4± 6.63	8.21
12	213	706.2±4.51	9.31	217	513.4±3.31	9.49	47	608.2± 6.61	7.46
13	210	735.5±4.81	9.47	211	534.8±3.36	9.14	43	643.7± 7.75	7.89
14	210	770.5±4.77	8.98	214	556.5±3.57	9.39	43	679.9± 8.59	8.29
15	209	805.6±4.81	8.63	212	579.3±3.77	9.49	43	720.3± 9.02	8.22
16	206	841.5±5.03	8.58	209	606.5±4.04	9.62	43	753.3± 9.22	8.03
17	206	876.4±5.19	8.50	206	631.2±4.33	9.84	41	785.9±10.10	8.23
18	206	913.5±5.51	8.66	205	655.3±4.41	9.65	38	824.7±10.53	7.87

TABLE 2.—*Standards of skeletal growth for purebred dairy calves*

HOLSTEINS

Age, and item of measurement	Animals	Average measurement	Coefficient of variability	Age, and item of measurements	Animals	Average measurement	Coefficient of variability
	Number	*Centimeters*	*Percent*		*Number*	*Centimeters*	*Percent*
6 months:				12 months—Continued			
Height at withers	184	101.8±0.23	3.02	Width of hips	182	40.0±0.12	4.10
Width of fore chest	184	28.3±0.14	6.58	Total length	172	112.9±0.27	3.12
Depth of fore chest	184	46.9±0.14	4.12	18 months:			
Width of hips	184	30.5±0.12	5.10	Height at withers	173	126.4±0.22	2.30
Total length	172	92.3±0.29	4.11	Width of fore chest	173	40.7±0.19	6.19
12 months:				Depth of fore chest	173	64.5±0.16	3.22
Height at withers	182	117.9±0.21	2.40	Width of hips	173	45.7±0.13	3.72
Width of fore chest	182	36.4±0.17	6.20	Total length	163	124.0±0.30	3.04
Depth of fore chest	182	58.2±0.14	3.32				

JERSEYS

6 months:				12 months—Continued			
Height at withers	193	95.5±0.24	3.48	Width of hips	198	36.3±0.11	4.10
Width of fore chest	193	23.4±0.14	8.08	Total length	170	106.0±0.24	2.97
Depth of fore chest	193	44.4±0.13	4.09	18 months:			
Width of hips	193	27.4±0.11	5.55	Height at withers	191	117.2±0.20	2.31
Total length	164	87.4±0.09	1.35	Width of fore chest	191	34.4±0.17	6.74
12 months:				Depth of fore chest	191	60.8±0.13	3.03
Height at withers	198	110.0±0.20	2.57	Width of hips	191	41.2±0.13	4.28
Width of fore chest	198	30.8±0.16	7.17	Total length	165	115.6±0.27	2.99
Depth of fore chest	198	55.1±0.12	2.94				

RED DANES

6 months:				12 months—Continued			
Height at withers	58	97.5±0.38	2.95	Width of hips	60	38.4±0.19	3.87
Width of fore chest	58	27.6±0.21	5.91	Total length	60	107.3±0.38	2.74
Depth of fore chest	58	45.8±0.21	3.51	18 months:			
Width of hips	58	29.9±0.20	5.03	Height at withers	57	118.8±0.36	2.30
Total length	58	90.0±0.50	4.19	Width of fore chest	57	38.3±0.32	6.25
12 months:				Depth of fore chest	57	61.6±0.24	2.88
Height at withers	60	110.9±0.31	2.18	Width of hips	57	44.8±0.26	4.38
Width of fore chest	60	33.8±0.27	6.15	Total length	57	117.8±0.41	2.60
Depth of fore chest	60	55.5±0.21	2.88				

The groups of crossbreds are relatively small; therefore, a statistical test is necessary to determine the significance of any apparent evidence of heterosis. For this purpose Student's *t* test as described by Snedecor [2] is employed, using as a hypothesis, or expected value, the mean between the two parent breed averages for outcross females for each weight or measurement. This mean hereafter will be referred to as the "expected" weight or measurement. Thus, this test is simply a measure of the significance of the difference between the crossbred averages and the mean of the two parent breed averages.

GROWTH IN LIVE WEIGHT

The data on live weight are given in tables 3, 4, and 5, and represent three different two-breed combinations.

TABLE 3.—*Live weight data on Jersey × Holstein calves and Holstein × Jersey calves*

CALVES FROM HOLSTEIN DAMS

Age (months)	Animals	Average weight (A)	Coefficient of variability	Average weight of dams	Mean of parent breed averages (B)	Difference (A−B)
	Number	*Pounds*	*Percent*	*Pounds*	*Pounds*	*Pounds*
Birth	8	76.6±2.63	9.71	86.0±3.14	75.5	1.1
1	8	99.6±4.01	11.38	115.9±3.97	95.1	4.5
2	8	128.9±2.71	5.95	157.1±5.41	129.9	−1.0
3	8	175.9±2.67	4.30	203.0±6.65	174.0	1.9
4	8	225.5±4.77	5.99	254.7±8.15	223.4	2.1
5	8	276.9±7.53	7.70	311.0±8.59	278.5	−1.6
6	8	330.4±7.55	6.47	370.7±9.99	333.0	−2.6
7	8	387.1±9.88	7.22	428.0±8.68	386.1	1.0
8	8	432.1±9.67	6.47	484.1±9.94	434.0	−1.9
9	8	473.9±12.87	7.69	532.4±7.52	478.9	−5.0
10	8	511.6±14.80	8.19	577.9±12.85	523.0	−11.4
11	8	548.6±15.14	7.81	620.6±14.64	566.2	−17.6
12	8	594.8±13.61	6.48	660.0±22.67	609.8	−15.0
13	8	621.0±13.96	6.36	723.0±14.03	635.2	−14.2
14	8	648.0±13.12	5.73	754.6±14.85	663.5	−15.5
15	8	687.5±7.67	3.16	782.6±10.16	692.5	−5.0
16	8	711.5±8.26	3.28	816.0±10.13	724.0	−12.5
17	8	742.0±13.41	5.12	846.3±17.69	753.8	−11.8
18	8	785.5±12.60	4.54	858.7±18.18	784.4	1.1

CALVES FROM JERSEY DAMS

Birth	10	74.9±2.47	10.44	51.9±2.19	75.5	−0.6
1	10	93.5±2.57	8.67	68.0±2.30	95.1	−1.6
2	10	129.0±3.16	7.74	92.3±3.54	129.9	−.9
3	10	179.9±5.02	8.81	123.3±4.49	174.0	5.9
4	10	238.5±5.32	7.05	159.6±5.57	223.4	15.1*
5	10	299.0±5.36	5.67	200.3±7.54	278.5	20.5**
6	10	356.7±4.52	4.01	240.7±9.84	333.0	23.7**
7	10	416.1±5.51	4.19	281.3±12.36	386.1	30.0**
8	10	460.2±7.21	4.95	310.6±14.18	434.0	26.2**
9	10	496.0±7.55	4.81	337.6±17.41	478.9	17.1*
10	10	543.8±10.28	5.98	366.0±18.90	523.0	20.8
11	10	578.9±13.42	7.32	395.6±23.20	566.2	12.7
12	10	629.4±12.91	6.48	431.3±23.01	609.8	19.6
13	10	666.8±14.14	6.70	458.9±19.58	635.2	31.6
14	10	687.8±12.58	5.78	481.7±20.12	663.5	24.3
15	10	717.0±15.17	6.69	497.3±26.46	692.5	24.5
16	10	742.7±13.90	5.91	534.9±24.58	724.0	18.7
17	10	790.6±18.21	7.28	547.3±28.18	753.8	36.8
18	10	822.2±16.10	6.19	574.1±22.88	784.4	37.8*

*Significant difference. **Highly significant difference.

[2] SNEDECOR, G. W. STATISTICAL METHODS. Ed. 4, Rev. ed., 485 pp. illus. Ames, Iowa, 1946.

TABLE 4.—*Live weight data on Red Dane × Holstein calves and on Holstein × Red Dane calves*

Age (months)	Animals	Average weight (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
	Number	*Pounds*	*Percent*	*Pounds*	*Pounds*
Birth	13	94.0±3.06	11.77	88.3	5.7
1	12	113.3±3.07	9.39	112.6	.7
2	13	154.0±4.20	9.84	152.1	1.9
3	13	206.2±4.84	8.48	201.9	4.3
4	13	260.9±4.04	5.59	256.5	4.4
5	13	316.5±5.17	5.89	314.7	1.8
6	13	368.3±5.77	5.65	370.6	−2.3
7	13	413.8±6.43	5.61	423.4	−9.6
8	11	466.6±9.82	6.99	470.0	−3.4
9	11	520.6±10.58	6.75	516.5	4.1
10	10	571.9±13.22	7.31	563.0	8.9
11	10	620.7±11.75	5.98	607.2	13.5
12	10	678.6±11.38	5.30	657.2	21.4
13	10	714.3±12.01	5.31	689.6	24.7
14	10	744.0±14.44	6.13	725.2	18.8
15	10	772.8±9.80	4.01	763.0	9.8
16	10	802.4±10.51	4.14	797.4	5.0
17	9	833.6±16.81	6.05	831.1	2.5
18	8	877.0±14.36	4.63	869.1	7.9

TABLE 5.—*Live weight data on Red Dane × Jersey calves and on Jersey × Red Dane calves*

Age (months)	Animals	Average weight (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
	Number	*Pounds*	*Percent*	*Pounds*	*Pounds*
Birth	8	70.5±2.95	11.38	68.0	2.5
1	8	90.1±3.71	11.66	89.1	1.0
2	8	128.0±4.74	10.48	122.6	5.4
3	8	174.9±6.48	10.49	164.8	10.1
4	8	223.4±7.89	9.89	211.7	11.7
5	8	276.8±8.30	8.49	262.5	14.3
6	8	329.6±8.53	7.32	312.7	16.9
7	8	380.8±10.54	7.83	360.4	20.4
8	8	428.4±13.81	9.13	401.3	27.1
9	8	468.6±13.88	8.38	441.9	26.7
10	8	511.1±15.41	8.53	480.9	30.2
11	8	546.9±20.04	10.37	518.4	28.5
12	8	590.9±19.89	9.53	560.8	30.1
13	8	618.5±22.34	10.22	589.3	29.2
14	8	650.9±20.29	8.82	618.2	32.7
15	8	677.0±21.98	9.19	649.8	27.2
16	8	707.3±20.65	8.26	679.9	27.4
17	7	739.9±28.98	10.35	708.5	31.4
18	7	765.7±28.62	9.90	740.0	25.7

The first point of interest in these tables is the birth weight of the crossbred calves. Apparently there is not enough difference in size between the parent breeds used here to exhibit the phenomenon described by Hammond,[3] where the size of the dam controls the size of the offspring. In the combinations representing the Holstein and Jersey breeds, in which there is the greatest contrast in size of the parent breeds, the female calves from Holstein sires and Jersey dams

[3] HAMMOND, J. PHYSIOLOGICAL FACTORS AFFECTING BIRTH WEIGHT. Nutr. Soc. Proc. 2: 8–12, illus. 1944.

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had an average birth weight of 74.9 pounds and those from Jersey sires and Holstein dams averaged 76.6 pounds, whereas the mean of the birth weight averages for the parent breeds was 75.5 pounds (table 3).

There was somewhat more difference in birth weight between the Jersey×Holstein males and the Holstein×Jersey males than between females of the same crosses. Seven males from Holstein sires and Jersey dams averaged 76.3±2.37 pounds at birth, and six males from Jersey sires and Holstein dams averaged 82.0±3.87 pounds. This difference is not statistically significant.

It is interesting to note further that the Holstein×Jersey calves from Jersey dams developed more rapidly after 3 months of age than did the reciprocal cross from Holstein dams (table 3).

In general it can be said that the cross breds have a tendency to be slightly heavier than the expected weight. This difference is not statistically significant in a majority of cases.

Reports of previous cross-breeding work[4,5] indicate there is more evidence of heterosis when two large breeds are crossed than when a large breed and a small one are crossed. The work at Beltsville has not borne out this theory. The crossbreds from the two largest breeds, the Holstein and the Red Dane, show the least average increase over the expected weight of any of the crosses. At 6, 7, and 8 months of age, the Holstein×Red Dane crossbreds averaged slightly less than the expected weight.

The figures show some interesting breed interactions in live weight. In the Holstein×Jersey crossbreds (table 3) the period of most rapid growth is from 4 to 8 months. In the Red Dane×Jersey crossbreds (table 5) growth seems to be quite uniform from the second month on. The Guernsey crosses afford the most striking examples of different breed interactions; however, these are treated separately in another section.

The coefficients of variability indicate that there is somewhat less variance in the crossbred groups than in the large purebred groups from which the weight standards were calculated.

SKELETAL GROWTH

The data on skeletal growth are given in tables 6, 7, and 8. Crosses involving Guernseys are omitted from this phase of the study because there was no suitable set of standards for purebred Guernseys.

[4] HAMMOND, J. ON THE RELATIVE GROWTH AND DEVELOPMENT OF VARIOUS BREEDS AND CROSSES OF CATTLE. Jour. Agr. Sci. [England] 10: [233]–289. 1920.

[5] COLE, L. J. THE WISCONSIN EXPERIMENT IN CROSSBREEDING CATTLE. World's Dairy Cong. Proc., Washington, Philadelphia, and Syracuse, 1923, 2: 1383–1388. 1924.

TABLE 6.—*Skeletal growth data on Jersey × Holstein calves and on Holstein × Jersey calves*

CALVES FROM HOLSTEIN DAMS

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
	Number	*Centimeters*	*Percent*	*Centimeters*	*Centimeters*
6 months:					
Height at withers	8	99.0±.65	1.86	98.6	0.4
Width of fore chest	8	26.4±.29	3.10	25.8	.6
Depth of fore chest	8	45.8±.45	2.80	45.7	.1
Width of hips	8	29.6±.33	3.19	28.9	.7
Total length	8	91.6±.88	2.71	89.9	1.7
12 months:					
Height at withers	8	112.8±.86	2.17	114.0	−1.2
Width of fore chest	8	32.4±.66	5.76	33.6	−1.2
Depth of fore chest	8	55.7±.62	3.13	56.7	−1.0
Width of hips	8	38.8±.61	4.47	38.2	.6
Total length	8	110.2±.80	2.05	109.4	.8
18 months:					
Height at withers	8	120.6±.83	1.94	121.8	−1.2
Width of fore chest	8	36.2±.70	5.50	37.5	−1.3
Depth of fore chest	8	61.5±.52	2.41	62.7	−1.2
Width of hips	8	44.2±.57	3.63	43.5	.7
Total length	8	120.7±1.20	2.80	119.8	.9

CALVES FROM JERSEY DAMS

6 months:					
Height at withers	10	100.4±.70	2.20	98.6	1.8*
Width of fore chest	10	26.3±.39	4.66	25.8	.5
Depth of fore chest	10	46.8±.30	2.02	45.7	1.1**
Width of hips	10	29.8±.29	3.03	28.9	.9*
Total length	10	93.4±.92	3.11	89.9	3.5**
12 months:					
Height at withers	10	115.7±.94	2.57	114.0	1.7
Width of fore chest	10	33.3±.32	3.05	33.6	−.3
Depth of fore chest	10	57.4±.42	2.32	56.7	.7
Width of hips	10	39.4±.28	2.26	38.2	1.2**
Total length	10	112.9±1.18	3.31	109.4	3.5*
18 months:					
Height at withers	10	123.5±1.09	2.79	121.8	1.7
Width of fore chest	10	38.0±.35	2.92	37.5	.5
Depth of fore chest	10	63.4±.49	2.43	62.7	.7
Width of hips	10	44.9±.48	3.36	43.5	1.4*
Total length	10	122.2±1.35	3.48	119.8	2.4

*Significant difference. **Highly significant difference.

TABLE 7.—*Skeletal growth data on Holstein × Red Dane calves and on Red Dane × Holstein calves*

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
6 months:	*Number*	*Centimeters*	*Percent*	*Centimeters*	*Centimeters*
Height at withers	13	100.8±.62	2.23	99.6	1.2
Width of fore chest	13	27.8±.39	5.01	27.9	−0.1
Depth of fore chest	13	46.8±.32	2.47	46.4	.4
Width of hips	13	30.2±.37	4.46	30.2	0
Total length	13	92.7±.72	2.82	91.2	1.5
12 months:					
Height at withers	10	115.1±.61	1.68	114.4	.7
Width of fore chest	10	35.1±.63	5.70	35.1	0
Depth of fore chest	10	57.2±.25	1.37	56.8	.4
Width of hips	10	39.8±.37	2.97	39.2	.6
Total length	10	110.8±.80	2.28	110.1	.7
18 months:					
Height at withers	10	122.8±.74	1.90	122.6	.2
Width of fore chest	10	39.7±.26	2.04	39.5	.2
Depth of fore chest	10	62.7±.49	2.46	63.0	−.3
Width of hips	10	45.6±.55	3.79	45.2	.4
Total length	10	121.8±.79	2.04	120.9	.9

TABLE 8.—*Skeletal growth data on calves by Red Dane sires and out of Jersey dams*

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
6 months:	*Number*	*Centimeters*	*Percent*	*Centimeters*	*Centimeters*
Height at withers	7	96.8±0.69	1.88	96.5	0.3
Width of fore chest	7	26.9±.61	6.02	25.5	1.4
Depth of fore chest	7	45.6±.42	2.46	45.1	.5
Width of hips	7	28.9±.63	5.79	28.7	.2
Total length	7	89.8±.69	2.03	88.7	1.1
12 months:					
Height at withers	8	110.6±1.19	3.04	110.5	.1
Width of fore chest	8	34.4±.77	6.48	32.3	1.1
Depth of fore chest	8	55.7±.47	2.40	55.2	.5
Width of hips	8	38.3±.62	4.57	37.3	1.0
Total length	8	107.8±1.11	2.92	106.6	1.2
18 months:					
Height at withers	7	118.4±1.46	3.27	118.0	.4
Width of fore chest	7	38.1±.70	4.85	36.3	1.8*
Depth of fore chest	7	61.6±.63	2.72	61.2	.4
Width of hips	7	44.3±.74	4.41	43.0	1.3
Total length	7	117.8±1.68	3.78	116.7	1.1

*Significant difference.

The same general trends are found in the skeletal measurements as in the live weight data. The most noticeable feature is that the Holstein × Jersey and Jersey × Holstein crossbreds seem to be somewhat rangier than the crossbreds of the other breeds, particularly at 6 months of age. The Holstein × Red Dane crossbreds show the least heterosis of any of the crossbreds. Again, as in live weight, the Red Dane × Jersey crossbreds are quite uniform in skeletal growth.

Figures 1 to 5 show the general conformation of the crossbreds resulting from various crosses. These photographs, all taken at 6 months of age, are of calves chosen as the most representative of their respective groups with regard to size and general growth habits.

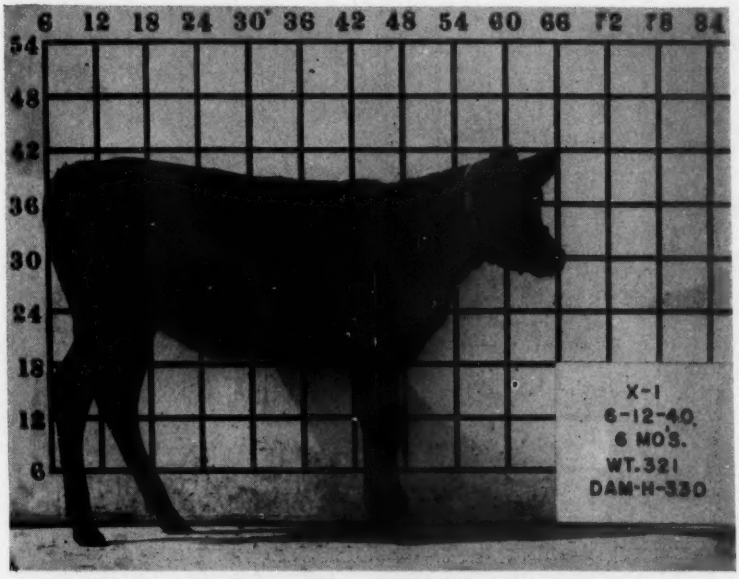


FIGURE 1.—Crossbred calf (Herd No. X-1) by a Jersey sire and out of a Holstein dam.

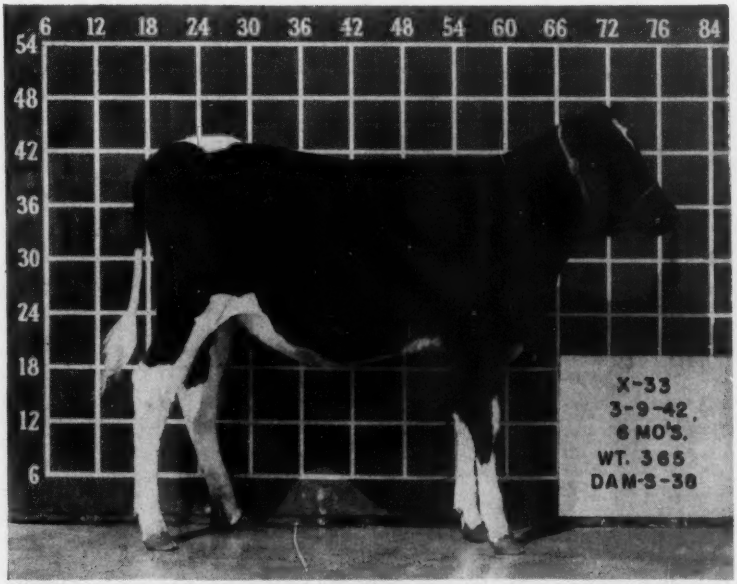


FIGURE 2.—Crossbred calf (Herd No. X-33) by a Holstein sire and out of a Guernsey dam.

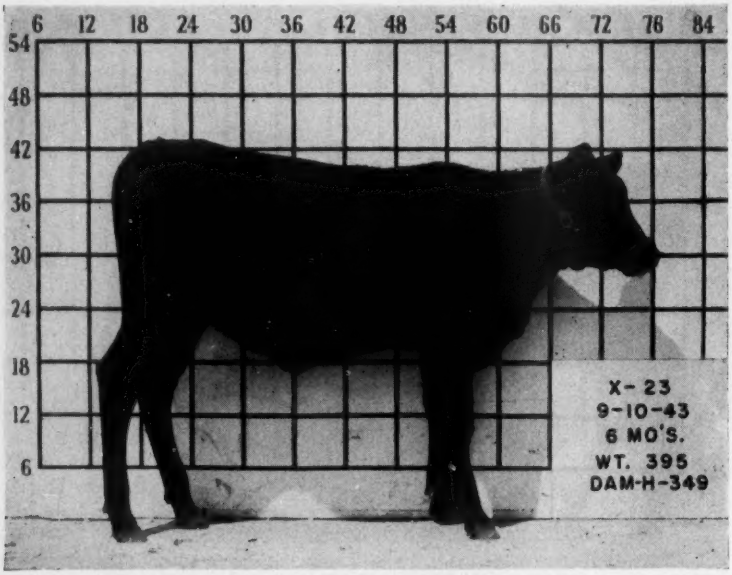


FIGURE 3.—Crossbred calf (Herd No. X-23) by a Holstein sire and out of a Red Dane dam.

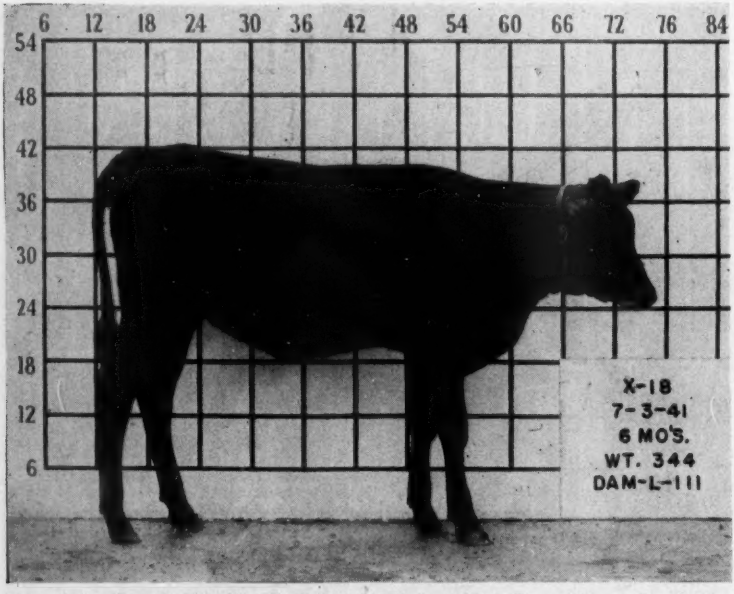
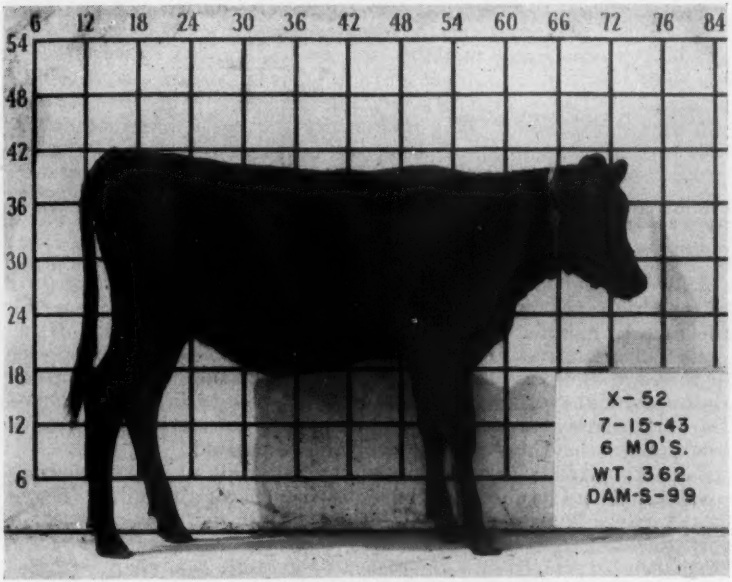


FIGURE 4.—Crossbred calf (Herd No. X-18) by a Red Dane sire and out of a Jersey dam.



FIRURE 5.—Crossbred calf (Herd No. X-52) by a Red Dane sire and out of a Guernsey dam.

GROWTH OF GUERNSEY CROSSES

The growth in live weight of the crosses involving Guernseys (Holstein × Guernsey and Red Dane × Guernsey) appears to follow a somewhat different pattern from that of the other crosses discussed thus far. For this reason an analysis of the data for these crosses seems desirable, even though no suitable standard for purebred Guernseys is available for comparison.

A study of the data indicated that the slope of the growth curve for the Holstein × Guernsey crossbreds differed from that of the Red Dane × Guernsey crossbreds; nor did these curves slope as might be expected judging from the data on the purebred Holsteins and Red Danes. To illustrate this, a calculation was made showing the percentage of the final weight gained during each month from birth to 18 months of age. As used here the "final weight" is the mean of the weights at 17, 18, and 19 months of age, which reduces the chance of error that could be introduced by using a single 18-month average weight. These calculations were made for the Holstein × Guernsey and Red Dane × Guernsey crossbreds and purebred Holstein and Red Dane groups. The figures are summarized in table 9.

TABLE 9.—*Comparative rate of growth of Holstein × Guernsey calves and Red Dane × Guernsey calves*

Period of growth (average)	Proportion of total growth per month			
	Holstein	Red Dane	Holstein × Guernsey	Red Dane × Guernsey
	Percent	*Percent*	*Percent*	*Percent*
Birth to 12 months	5.62	5.42	5.34	5.52
12 to 18 months	3.70	4.22	4.35	3.68

It will be noted that in the purebred groups the rate of growth of the Holsteins was relatively higher than that of the Red Danes for the first 12 months, whereas from 12 to 18 months the Red Danes gained at a relatively faster rate than did the Holsteins. When each of these two breeds was crossed with Guernseys the trend was exactly the opposite, the higher early rate of growth being attained by the Red Dane×Guernsey crossbreds while the Holstein×Guernsey crossbreds grow relatively faster during the later period.

The total growth for the two periods also affords an interesting comparison. The Holsteins gained 67.47 percent of their final weight from birth to 12 months, and 22.20 percent from 12 to 18 months. The corresponding figures for the other groups are: Red Danes, 64.99 and 25.31 percent; Holstein×Guernsey, 64.12 and 25.90 percent; Red Dane×Guernsey, 66.21 and 22.05 percent. It will be noted that the percentages for the Red Dane×Guernsey crossbreds closely approach those of the purebred Holsteins, while the Holstein×Guernsey figures more nearly approximate those of the purebred Red Danes.

Still another method may be used to illustrate these different breed interactions. For this method, the assumption is made from the data in tables 3, 4, and 5 that the weights of the crossbreds will fall approximately midway between the parent breed averages. On the basis of this assumption, the Guernsey average weights can be roughly estimated from the weights of the crossbreds and the average weights of the other parent breed. Table 10 shows the estimates of the Guernsey average weights made from the data on the crosses with Holsteins and with Red Danes.

TABLE 10.—*Estimate of Guernsey weight averages*

Age (months)	Estimate of Guernsey weight averages	
	From Holstein × Guernsey crossbreds	From Red Dane × Guernsey crossbreds
	Pounds	*Pounds*
Birth	66	91
6	260	330
12	512	616
18	739	734

The wide divergence of these estimates makes it obvious that the assumption made above is not valid in the case of these crosses. The

relatively rapid rate of early growth in the Red Dane×Guernsey crossbreds is again apparent in this tabulation.

We have no explanation for the different breed interactions described here. Although the figures presented in tables 9 and 10 do not lend themselves to statistical analysis, the data have been explored thoroughly, and it seems very unlikely that the differences are due entirely to chance variation in the small groups of crossbreds. Probably there will be no satisfactory explanation for these breed interactions until much more is known about cattle genetics.

The data in this section indicate that the growth obtained by crossing two given breeds does not necessarily afford an accurate prediction of the result of crossing either breed with other breeds.

SUMMARY

Growth studies are presented on first-generation crossbred dairy calves, representing crosses of Holstein, Jersey, Guernsey, and Red Dane cattle. The measures of growth used are live weight and a set of five skeletal measurements.

There is some indication that the crossbreds tend to be slightly larger than the mean of the parent breed averages. Heterosis is indicated in some groups by the fact that the crossbreds are significantly larger than the expected weight or measurement, but this does not hold true for all crossbred calves.

Some interesting differences in breed interactions are shown, particularly in the Guernsey crosses.

THE EFFECTS OF AIRPLANE DDT APPLICATIONS ON CITRUS GROVES IN FLORIDA[1] [2]

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INTRODUCTION

During the war years the Army and Navy made extensive use of airplanes for the dispersal of DDT for mosquito and fly control. Linquist *et al.* (*1*) [4] stated that a 5-percent solution of DDT applied at 2–3 quarts per acre would give satisfactory adult mosquito control. As the result of both experimental and practical experience, counties and cities have begun to engage in this type of pest-control program. DDT has shown itself to be most beneficial when used in this manner. However, instances have been reported where DDT apparently was having some detrimental effects.

Griffiths and Thompson (*2*) and Griffiths and Stearns (*3*) reported a reduction in the parasites and predators of Florida red scale (*Chrysomphalus aonidum* (L.)) and a subsequent abnormal population increase for this pest where DDT was applied with conventional spray equipment on citrus trees in Florida. Woglum (*4*) reported complications in citricola scale (*Coccus pseudomagnoliarum* (Kuw.)) and cottony-cushion scale (*Icerya purchasi* Mask) control in California citrus groves. More recently Morrill and Otanes (*5*) have reported scale and mealybug infestations in and around Manila which they believed were attributable to the regular application of DDT over that city. Consequently, the widespread use of airplane dispersed DDT in certain coastal areas of Florida was viewed with apprehension by some citrus growers.

EXPERIMENTAL PROCEDURE

The following is an account of observations made in citrus groves in and around Cocoa in Brevard County on the east cost of Florida. Here, the county had undertaken an extensive campaign for mosquito control. This area was typical of others in Florida where DDT was

[1] Received for publication June 2, 1948, Journal of Agricultural Research, Washington, D. C.
[2] The authors wish to take this opportunity to thank Lt. Comdr. John M. Hirst of the U. S. Navy and Thomas L. Cain, Jr., of Cocoa, Fla., for their help and cooperation.
[3] Entomologist and Insecticide Chemist at the Citrus Experiment Station, Lake Alfred, Fla.
[4] Italic numbers in parentheses refer to Literature Cited, p. 476.

sprayed from airplanes, and it is believed that the observations made here would be applicable to other citrus growing areas of the State.

In the Brevard County operations, flights were scheduled according to "bite counts" so that some areas were more frequently sprayed than others. The material used consisted of 35 pounds of technical DDT per 100 gallons of spray and this was dispersed at about 10 gallons per minute by a plane that averaged 70 miles per hour. As nearly as possible an attempt was made to spread the material over approximately a 100-foot swath. With these figures as a basis, it may be calculated that about one quarter of a pound of DDT was distributed over an acre area. If it is assumed that this was an absolutely flat surface, then less than 2.8 μg of DDT was deposited per cm^2. However, since an acre of vegetative growth comprises many acres of surface, only an infinitesimally small amount of actual DDT would be expected to be deposited on any surface at any application.

Determinations of the actual amounts of DDT present following the airplane sprayings were made according to Redd's modification of a method by Schecter et al. (*6*). This method determines the total amount of DDT present both inside and on the surface of the leaf. The procedure included the following technique: Twenty-five leaves were selected at random and a round disk was punched from each leaf. Each disk represented an area of 1 square inch (i. e., a total leaf surface, upper and lower, of 2 square inches). These disks were extracted for 1 hour with 150 ml. of petroleum either (boiling point 35° to 60° C.) In a Soxhlet extractor. The volume was reduced by evaporation to 15 ml. and the material transferred to a 50-ml. centrifuge tube.

The extraction flask was washed twice with small portions of the ether and the washings were added to the centrifuge tube. The ether was evaporated, and the tubes cooled in water. Five ml. of nitrating mixture composed of 1 to 1 volume of fuming nitric acid (specific gravity 1.50) and concentrated sulfuric acid (specific gravity 1.84) were added, and the tubes were heated in a water bath for 1 hour at 100° C. After cooling the tubes in cold water, 25 ml. of ice water were added. The solution was transferred to a 60-ml. separatory funnel, and after the tubes had been thoroughly washed, the wash was added to the funnel. This mixture was then extracted with 20 ml. of a solution containing 60-percent petroleum ether and 40-percent benzene. It was shaken vigorously for 1 minute and after separation of the layers, the aqueous portion was discarded. The solution was washed with 5 ml. of water and the aqueous portion discarded. After a second washing with 10 ml. of 2-percent sodium hydroxide the aqueous portion was again discarded.

Following this a third washing with water was performed. The petroleum etherbenzene portion was then transferred to a dry flask, placed in a water bath and evaporated to dryness under vacuum. Five ml. of anhydrous benzene and 10 ml. of sodium methylate solution (10 gr. $\pm$ 0.1 gr. per 100 ml. of anhydrous methanol) were added to the flask and thoroughly mixed. The blue color was read within 15 minutes and the amount of p,p'-DDT was calculated from standard-curves prepared from pure tetranitro p,p'-DDT using 590-mμ filter.

Ebling (7) reported that DDT in kerosene emulsions was absorbed into the citrus leaf. In order to check this report under Florida conditions a procedure was worked out to determine whether DDT was absorbed by the leaves. It was found that when DDT and oil emulsions were sprayed on citrus trees some DDT was absorbed by the leaf. The absorbed DDT was determined in the following manner: Twenty-five leaves selected at random were placed in a 1-percent solution of Nytron, a highly active surface agent, and thoroughly scrubbed with a soft brush. Following this treatment they were rinsed several times with tap water and with distilled water and allowed to air dry. The leaves were then analyzed for DDT by the procedure described above. The DDT found at this analysis was considered to have been absorbed into the inside of the leaf. Any difference between this amount and the total amount found on a similar sample which had not been washed was considered to represent the DDT on the surface of the leaf.

RESULTS

Table 1 presents data on p,p'-DDT deposits on citrus foliage at various locations and various dates during the 1947 mosquito spraying season. In some cases the DDT is expressed both as internal and total DDT. The part of the tree from which the samples were taken is indicated as well as the number of times sprayed by county planes and the date of the last spraying.

Several facts are evident from table 1. All DDT deposits were within a range where experimental error was as great as the differences noted. Thus, any figure under 0.5 μg DDT per cm^2 is not a reliable one. However, certain trends are evident. On August 18 and 19 samples were taken before and after an application. The trees from which these samples were taken were located inside the city limits of Cocoa and in this area heavier than average applications occurred. Apparently about 0.10–0.15 μg DDT was deposited per cm^2. Reference to the entire set of figures indicates that although this amount might be applied at one application, in no instance did the total amount on the leaf accumulate to as much as 0.3 μg per cm^2. Apparently the dissipation between sprayings was as great or greater than the amount deposited. The data of November 22 show marked irregularity, but this is easily explained by the low levels of DDT being considered. However, these data appear to indicate that most of the DDT at that date was internal. In any case, in spite of as many as 10 applications, DDT was still present in only minute amounts. Griffiths and Stearns stated that damage to the predaceous and parasitic insects that helped control Florida red scale was accomplished when DDT deposits were higher than 0.5 μg cm^2. Thus, in no instance did deposits attain a level which would be expected to adversely affect such beneficial populations.

TABLE 1.—*DDT deposits on citrus foliage following airplane applications, 1947*

Date	Grove	Location on tree	μg of DDT per cm²		Number of times sprayed by county planes	Date of last spray
			Internal	Total		
May 26	A–1	Top		0	0	
	A–2	do		0	0	
	A–3	do		0	0	
	B	Side		0	0	
	C	do		0	0	
July 17	A–1	Top		0.13	4	July 12
Aug. 12	do	do		.12	6	Aug. 8
	do	Side		.11	6	Do.
	A–2	Top		.06	6	Do.
	do	Side		.06	6	Do.
	A–3	Top		.06	6	Do.
	do	Side		.06	6	Do.
Aug. 18	D	Top		.17	4	July 16
	do	Side		.12	4	Do.
Aug. 19	D	Top		.28	5	Aug. 19
	do	Side		.28	5	Do.
Nov. 22	A–1	Top	0.09	.12	10	Oct. 21
	A–3	do	.04	.03	10	Do.
	B	Side		.12	10	Oct. 30
	C	do		.03	10	Do.
	D	Top	.18	.10	7	Oct. 9
	E	do	.09	.03	7	Do.
	F	do	.12	.06	7	Do.

Notes on parasitic and predaceous populations were made during the summer and fall in groves sprayed with DDT. Grove A is of particular interest. This grove was located on Merritt Island adjacent to Indian River. The owner lived on the grove and the trees about his house received considerably more DDT than did those at the far side of the grove. Samples labeled A–1 in table 1 were taken adjacent to the house. A–3 was at the far side of the grove and A–2 was midway between A–1 and A–3. In August, counts of Florida red scale parasites were made both at A–1 and A–3 locations. No parasites were recorded. At the same time more twice-stabbed lady beetles (*Chilocoris stigma* (Say)) appeared to be present at A–3 than at A–1 location and there was definitely more evidence of feeding holes in red scale armors at A–3 than A–1. Florida red scale was common, but not abundant, at both locations. The fact that no parasites were found in the sample examined is probably of no significance, since other samples taken from the east coast of Florida during July and August failed to show any parasites either. Red scale populations were generally low at that time and apparently parasite density was correspondingly low. In September, samples were again taken and this time one second stage parasite (presumably *Prospaltella aurantii* (How.)) was found.

A final survey was made on November 22 in groves A, D, and F. Evidences of parasitism were recorded as shown in table 2. No third stage parasites were found, but this was again in line with other groves sampled in other places on the east coast of the State. Second-stage parasitism was common and well within the range of normal expectancy for that time of the year. Not only were the parasites common, but twice-stabbed lady beetles were present and often numerous at all locations noted in table 1 for the November date of sampling.

TABLE 2.—*Parasitism of Florida red scale in November after 10 applications of DDT*

Grove	Location on tree	Second stage parasites		Third stage parasites	μg of DDT for cm²
		Live parasites,/100 live scale [a]	Evidences of parasites/[b] 100 live scale		
D	Top	4	10	0	0.10
A–1	do	12	24	0	.12
A–1	Side	14	16	0	.12
A–3	Top	24	32	0	.04
F	do	20	20	0	.06

[a] Live scale included only late second- and very early third-stage females.
[b] Evidences of parasites included live larvae or pupae, dead ones, and emergence holes.

DISCUSSION

In considering the possible deleterious effects of airplane DDT spraying on Florida red scale control, several factors should be kept in mind. Apparently only small amounts of DDT were deposited on citrus foliage at any one application. These amounts were largely dissipated between applications so that there was no accumulation of DDT on the leaves. At the end of the spray season, there was no more DDT than early in the year, and in the fall most of the insecticide appeared to be on the inside of the leaf. At no time during the season was more than 0.3 μg per cm² recorded. This amount would not be considered as harmful to the insects which are parasitic or predaceous upon Florida red scale.

Observations on parasites and predators substantiated this conclusion since normal concentrations of lady beetles and parasites of second stage scale were found at the end of the season in November. The fact that no parasites of third stage scale were recorded may be discounted since the incidence of this parasite was low elsewhere along the east coast of Florida.

The only possibilities for detrimental effects from DDT would appear to be where an overzealous pilot regularly applied too much DDT to some area. Even then it would probably be difficult to get a deposit of DDT sufficiently high to adversely affect the insects that feed in or upon Florida red scale. This is evidenced by the fact that locations A–1, D, E, and F in table I were regularly sprayed with more than the specified amount of DDT. These facts would appear to justify the conclusion that DDT airplane spraying for mosquito control in Brevard County, Fla., was not detrimental to insect parasites and predators of Florida red scale in citrus groves and that from this standpoint, groves could be treated without fear of complications in red scale control.

SUMMARY AND CONCLUSIONS

In 1947 in Brevard County, Fla., airplanes were used extensively to distribute DDT for mosquito control. A study of the amount of p,p′DDT deposited on citrus foliage and of the effects of this DDT on the parasites and predators of Florida red scale in citrus groves

was made. No deposits of more than 0.3 μg of DDT per cm² were found and in November, parasitic and predaceous populations were apparently normal. Most of the DDT retained by the foliage appeared to have been absorbed into the leaf tissue. It is concluded that the spraying of DDT as practiced in Brevard County would have no detrimental effects on Florida red scale control in citrus groves.

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PALATABILITY TESTS ON POTATOES GROWN IN SOIL TREATED WITH THE INSECTICIDES BENZENE HEXACHLORIDE, CHLORDANE, AND CHLORINATED CAMPHENE [1]

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INTRODUCTION

The effectiveness of soil treatment with benzene hexachloride for the control of wireworm has been demonstrated at the Connecticut Agricultural Experiment Station.[2] Potato crops in Connecticut and other States are subject to considerable damage from wireworm, and the use of an effective insecticide would mean a substantial increase in the annual marketable yield.

Potatoes grown in soil treated with benzene hexachloride have an off-flavor according to numerous local consumers. The School of Home Economics at the University of Connecticut was asked to conduct palatability tests to determine whether the comments on off-flavor were justified. The first test, in the spring of 1947, showed that, at certain dosages, benzene hexachloride imparted a foreign flavor in the cooked potatoes.[3] The palatability tests were also carried out on the 1947 crop. The results of these tests are reported in this paper. In addition, these latter tests included potatoes grown in soil treated with chlorinated camphene, and in soil treated with chlordane. These chemicals were both being studied as insecticides. The purpose of these palatability tests was to determine whether the various insecticide treatments used for wireworm control affected the flavor of cooked potatoes.

PROCEDURE

Treated and untreated samples of potatoes, from both experimental and commercial fields, were supplied by the Connecticut Station. The tubers were stored in a dark basement room until used. The palatability panel for the taste tests consisted of staff members and students at the University of Connecticut.

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[1] Received for publication September 7, 1948. The authors acknowledge the aid of C. I. Bliss, Biometrician, Connecticut Agricultural Experiment Station; Martha Potgieter, Associate Professor of Foods and Nutrition, University of Connecticut; and all those who acted as tasters throughout the experiment.

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A uniform method of cooking was used in the nine tests. Two hundred gm. of peeled potatoes were cut into ⅛-inch slices and cooked in 300 gm. of boiling water until tender. The degree of doneness was determined with a cake tester and the cooking time averaged 15 to 20 minutes for all samples. Although parts of four or five potatoes were used for each sample, some of the inconsistencies observed may have been due to too limited sampling. After draining, they were placed on marked plates, mashed with a fork, and covered.

The tasting for all but two of the tests (Nos. 4 and 5) was done in this manner. The samples for each day were lettered and arranged in a random order. The tasters indicated the presence or absence of any off-flavor by plus (+) and minus (−) signs, respectively, and its intensity by (+), (++), etc. They also were asked to describe the flavor. Reference samples, identified as treated and untreated, were available to indicate to the tasters the nature of the off-flavor. In test No. 4 the samples were placed in order of the increasing dosage of the insecticide. The tasters were asked simply to check the presence or absence of any off-flavor. In test No. 5 the tasters were given eight samples (four treated and four untreated), arranged in random order, and were asked to group them into two groups of four identical samples. The potato samples tested are listed and described in table 1.

TABLE 1.—*Description of Potatoes Used in Palatability Tests*

Test No.	Variety	Insecticide	Dosage (pounds per acre)		Method of application
			Test samples	Reference samples	
1	Katahdin	Benzene hexachloride	0, 1, 2, 2½,[1] 8	0, 8	Spray.
2	do	do	0, 1, 2, 2½,[1] 8	0, 8	Do.
3	Green Mountain	Benzene hexachloride[2]	0, 7½, 50, 50	0	Dust.
4	do	do[2]	0, 7½, 50, 0		Do.
5	do	do[2]	0, 0, 0, 0, 50, 50, 50, 50		Do.
6	do	Pure gamma benzene hexachloride.	0, 0.25, 0.25, 0.25, 0.25	0. 25	Do.
7	Katahdin	Chlordane	0, 1, 2, 4, 8	0, 8	Spray.
8	do	Chlorinated camphene	0, 1, 2, 4, 8	0, 2	Do.
9	Green Mountain	do	0, 1, 1, 2, 2	0, 8	Do.

[1] Samples from commercial field.
[2] Soil treated in 1946; no treatment in 1947.

The method used to analyze the data depended upon the type of test. In the tests in which the subjects were asked to indicate the intensity of the off-flavor, the original markings were converted to numerical scores for analysis. These scores were those given by Fisher and Yates [4] for ranked data in series of 4 (−1.03, −0.30, 0.30, 1.03 in increasing order of off-flavor) or 5 (−1.16, −0.50, 0, 0.50, 1.16). Where two or more samples were marked alike, the corresponding scores were averaged. The scores were totaled for each sample and the statistical significance of the results in each test was assessed by an analysis of variance.

When only the presence or absence of an off-flavor was reported in a fixed order of tasting (test No. 4), the judgments on two successive

[4] FISHER, R. A., and YATES, F. STATISTICAL TABLES FOR BIOLOGICAL, AGRICULTURAL AND MEDICAL RESEARCH. Oliver and Boyd, Edinburgh. 1948.

samples were tested for independence by computing chi-square from 2×2 contingency tables, using Yates' correction for continuity. The proportion of off-flavor judgments in the totals for treated and untreated samples were then compared in a similar fashion. In test No. 5 in which eight samples were divided into two groups of four each, a frequency distribution was prepared of the observed number of correct judgments and compared by chi-square with the number to be expected if the subjects had been unable to distinguish between the treated and untreated potatoes.

An effort was made to classify the tasters on the basis of their consistency in duplicate tests Nos. 1 and 2 but the scores for any given taster differed more from day to day than those of different tasters on the same day. Since the tasters could not be classified as good or poor, all scores were used in determining the totals in the benzene hexachloride tests. All tasters' scores were included also in the totals for chlordane and chlorinated camphene.

RESULTS AND DISCUSSION

BENZENE HEXACHLORIDE

The scores for tests Nos. 1 and 2 (table 2) indicate that the greater the dosage of benzene hexachloride the stronger the off-flavor in the potatoes. The mean scores differ significantly from one another ($P < 0.001$) and show an increase in value (except at the 1- and 2-pound levels in test 1) as the dosage of the insecticide is increased.

The tasters who were able to detect the insecticide found the flavor quite objectionable. They described it as strong, earthy, metallic, bitter, medicinal, musty, and stinging or biting to the tongue. Some found that the flavor persisted as long as 2 or 3 hours.

TABLE 2.—*Total scores showing extent of off-flavor in potatoes grown in soil treated with benzene hexachloride at different dosages in pounds per acre*

Test No.	Number of tasters	Total scores at pounds-per-acre dosages of—				
		0	1	2	2½ [1]	8
1	16	−8.79	1.38	−5.65	5.69	7.36
2	27	−13.80	−6.96	5.34	6.71	8.75

[1] Sample from commercial field.

When benzene hexachloride was applied to the soil in the year prior to that in which the potatoes were grown, the results were less conclusive. In two tests Nos. 3 and 4 (tables 3 and 4), the tasters were able to distinguish between samples that had been grown on treated and untreated soil. In test No. 3 in which the random order of tasting was followed, the scores differ significantly ($P < 0.001$) from one another and are related to the dosage of the insecticide. The difference between the two scores at the 50-pound level probably indicates irregularity in the effect of the chemical on the individual potatoes. Hence, it would be advisable to take samples from a larger number of potatoes (a procedure which since has been adopted).

When the samples were tasted in a predetermined order (test No. 4), the tasters distinguished between the samples grown in treated and untreated soil ($P. < 0.001$) but apparently found no difference between the two dosages of insecticide ($P > 0.9$). The response to a given sample apparently was unaffected by the subject's response to the preceding sample. In contrast, when 4 samples from soil treated with 50 pounds per acre and 4 samples from untreated soil were compared (test No. 5, table 5), the distribution of correctly identified samples in the series of 8 might have occurred in 58 out of 10 cases in the absence of any ability to discriminate between treated and untreated samples. The poor quality of both the treated and untreated potatoes and the lack of reference samples may have made the judging more difficult in this case even though all of the tasters were experienced. Although the soil was treated the year before with benzene hexachloride, any off-flavor detected in the potatoes was

TABLE 3.—*Total scores showing extent of off-flavor in potatoes grown during 1947 in soil treated with benzene hexachloride in 1946 at different dosages in pounds per acre*

Test No.	Number of tasters	Total scores at pounds-per-acre dosages of—			
		0	7½	50	50
3	27	−10.61	−6.86	13.99	3.54

TABLE 4.—*Number of subjects reporting off-flavor present and absent in test No. 4, with potatoes grown in soil treated with benzene hexachloride in 1946. The samples were tasted by each subject in the order shown* [1]

Off-flavor rated as	Number of subjects detecting off-flavor with following order of tasting at pounds-per-acre dosages of—			
	0	7½	50	0
Present	6	15	13	6
Absent	14	5	7	14

[1] Treated vs. untreated: $X^2=11.25$, $n=1$, $P<0.001$
7½ lb. vs. 50 lb.: $X^2=0.120$, $n=1$, $P>0.9$

TABLE 5.—*Results in palatability test No. 5, consisting of 4 samples of potatoes grown during 1947 in soil t reated in 1946 with 50 pounds per acre of benzene hexachloride and 4 samples of potatoes from untreated soil* [1]

Number of samples correctly identified	Frequency	
	Observed	Expected
0		0.3 | 5.3
2	6	5.0 |
4	9	11.3
6	7	5.0 | 5.3
8		.3 |
Total	22	21.9

[1] Comparing observed and expected frequencies, $X^2=1.106$, $n=2$, $P=0.58$.

like that found in the crop grown the year the soil was treated. But, some of the tasters reported that the flavor was less marked.

The results of tests on the pure gamma isomer of benzene hexachloride are shown in table 6. The two samples of potatoes were collected from each of two plots which received the same treatment and from an untreated plot, all in the same field. As indicated by the significant mean square in the first row of the analysis of variance, the tasters distinguished the treated from the untreated potatoes. Although plots I and II were replicates, the potatoes from one plot tasted more strongly of the insecticide than those from the other. The tasters described the flavor, when detected correctly, as acid, strong, slightly earthy, or medicinal, terms similar to those used for benzene hexachloride. Several tasters found the flavor less objectionable than in former tests and others reported no after taste.

CHLORDANE

Although the scores for the different samples in the chlordane test (No. 7, table 7) differed significantly ($P<0.05$) from one another,

TABLE 6.—*Total scores for 25 subjects showing extent of off-flavor, in duplicate samples from 2 plots of potatoes both grown in soil treated with 0.25 pounds per acre of pure gamma benzene hexachloride, test No. 6, and the accompanying analysis of variance between the scores*

Item	Untreated	Plot I		Plot II	
		Sample 1	Sample	Sample 1	Sample 2
Total scores	−10.13	−3.91	−5.49	10.82	8.77

Source of variation	Degrees of freedom	Mean square	F
Untreated *vs.* treated	1	5.1430	[1] 9.44
Between treated plots	1	8.4042	[1] 15.4
Between samples within plots	2	.0670	
Remainder	96	.5450	

[1] Significant at $P<0.01$.

TABLE 7.—*Total scores showing extent of off-flavor in potatoes grown in soil treated with chlordane and with chlorinated camphene*

Soil treated with—	Test No.	Number of tasters	Total scores at pounds-per-acre dosages of—				
			0	1	2	4	8
Chlordane	7	28	−4.55	7.84	−8.49	7.34	−2.14
Chlorinated camphene	8	26	−4.22	−7.78	13.11	−1.76	.65
			Total scores at pounds-per-acre dosages of—				
			0	1	1	2	2
Do	9	23	−3.01	−10.65	2.07	11.01	.59

there was no apparent relation between the dose of insecticide and the intensity of off-flavor. Any off-flavor was described as metallic, musty, oily, or chemical. Several remarked that no sample had a strong or objectionable flavor.

CHLORINATED CAMPHENE

Table 7 also gives the total scores for tests Nos. 8 and 9. The relation between dosage and total score was not consistent in either test. Although scores for the different dosage levels differed significantly from one another, the average score for the treated samples did not differ from that for the untreated potatoes. Since the duplicates differed significantly from one another in test No. 9 it is apparent that factors other than the insecticide affected the flavor. The flavor was described as metallic, musty, oily, slightly old, tinny, and earthy. Some found the flavor not objectionable. Chlorinated camphene met with more disfavor, however, than did chlordane.

COMMENTS

Although the results from a single crop-year are not wholly conclusive, they are being reported because of their economic importance.

The commercial dosage for wireworm control for the insecticides tested is 2 pounds per acre. Greenwood [1] reported that benzene hexachloride applied as dust is effective at the rate of 2 to 2.5 pounds per acre when applied broadcast and at the rate of 1.32 pounds per acre as row treatment. Since benzene hexachloride could be detected at a level of 1 pound per acre, and since the flavor is particularly objectionable, its use as an insecticide for control of wireworm in potatoes cannot be recommended.

Further experimentation is needed before definite statements can be made in relation to chlordane. Variations in the chlordane scores indicate that broader sampling would be advisable. Further tests with chlorinated camphene also would be necessary before drawing conclusions as to its effect on the flavor of the potatoes, but it has been found to be ineffective in controlling the wireworm.

SUMMARY AND CONCLUSIONS

Palatability tests were conducted on the presence of an off-flavor in cooked potatoes grown in soil treated with either benzene hexachloride, chlordane, or chlorinated camphene for the control of wireworm.

In the case of benzene hexachloride, the intensity of the flavor increased with the dosage of insecticide and was detected even at the minimum level tested of 1 pound per acre. The flavor was particularly objectionable. Although inconclusive, the evidence suggests that potatoes grown in soil treated in the preceding year carried the same flavor. Apparently the pure gamma isomer of benzene hexachloride also imparted a foreign flavor to potatoes but the flavor was less objectionable than with the crude form.

Tests with potatoes grown in chlordane-treated soil were inconclusive but any resulting flavor from chlordane was much less objectionable than the flavor of benzene hexachloride.

The evidence for chlorinated camphene also was inconclusive. However, its flavor seemed to be more objectionable than that of chlordane.

THE DIGESTIBILITY AND METABOLIZABILITY BY LAMBS OF A STANDARD RATION OF ALFALFA AND CORN AND ONE CONTAINING COTTONSEED HULLS[1]

R. M. FORBES, Assistant Professor of animal husbandry, and W. P. GARRIGUS, chairman, Animal Industry Group Kentucky Agricultural Experiment Station

INTRODUCTION

In a large section of this country cottonseed hulls are fed extensively to cattle and sheep. Since hulls are extremely low in protein and are relatively unpalatable, cottonseed meal is frequently fed with them. Such rations may be adequate in protein and in energy, but will be deficient in calcium, carotene, and vitamin D.

Studies reported in this paper were made in the course of investigations whose object was to formulate nutritionally adequate rations containing significant quantities of cottonseed hulls and to compare them with a standard ration of alfalfa hay and corn, using sheep as experimental subjects.

REVIEW OF THE LITERATURE

The first reports dealing with the digestibility of cottonseed hulls were made by Harrington (*10*)[2] in 1891 and Emery and Kilgore (*2*). The Texas investigator (*10*) found that five steers digested 41.6, 5.7, 78.0, 30.4, and 41.2 percent respectively, of the dry matter protein, either extract, nitrogen-free extract and fiber. The North Carolina report (*2*) states that a cow in late lactation was given 21 pounds of cottonseed hulls daily but never completely consumed this amount. From a 4-day collection of feces digestion coefficients were calculated as follows: Dry matter 35.9, protein 24.6, ether extract 80.6, nitrogen-free extract 40.3, and crude fiber 27.1 percent. These early reports indicate that cottonseed hulls constitute an extremely low-grade feed.

Results of further investigations at the same institutions by Fraps (*9*) and by Emery and Kilgore (*3*), involving a cow, two goats, and five sheep, differed from those reported previously in that digestibility of protein was a negative value in all cases but one. The apparent digestibility of protein by a goat amounted to 1.2 percent.

The results obtained from these investigations represent the available data on the direct determination of digestibility of cottonseed

[1] The investigation reported in this paper was made as part of a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

[2] Italic numbers in parentheses refer to Literature Cited, p. 488.

Journal of Agricultural Research
Washington, D. C.

Vol. 78, No. 11
June 1, 1949
Key No. Ky.-25

hulls. The Texas and North Carolina stations have also computed digestibility by the indirect method: Fraps (7, 8, 9), Emery and Kilgore (2, 3, 4). One cow, 10 sheep and 9 steers were used in the experiments from which the computations were made. There was a consistently negative protein digestibility (12–61 percent) when the hulls were fed with alfalfa hay, with cottonseed meal and alfalfa hay, or with cottonseed meal alone. The average total digestible nutrients of cottonseed hulls as calculated by Schneider (*11*) from the data referred to above are as follows: For cattle 45.2 percent, and for sheep 52.8 percent on the dry basis. The digestible protein content of the hulls averages −0.6 percent, according to the same author.

EXPERIMENTAL PROCEDURE

In view of the obvious effect of associative digestibility exhibited by cottonseed hulls in earlier work, an effort was made to determine the digestibility and metabolizability of complete rations only.

The experimental animals were five wether-lambs bred on the experiment station farm. Throughout the experiment (fall of 1947) they were maintained in metabolism crates. The composition of rations fed is shown in table 1. Ration 1 was composed of 44.5 percent of good-quality chopped alfalfa hay and 55.5 percent of yellow corn. Ration 2 was composed of 50.6 percent of yellow corn, 15.5 percent of cottonseed meal, 4.0 percent of alfalfa leaf meal, and 29.9 percent of cottonseed hulls containing 10 percent of molasses. The rations were so compounded as to be nearly equal in gross nutrient content.

Sufficient feed was mixed and weighed in individual portions before each trial to last throughout a 10-day preliminary period and an 8-day collection period. During the latter period feces and urine were collected daily. Feces were preserved by drying the total daily samples, separately. At the end of the trial these samples were mixed, ground, and sampled carefully for analysis. An aliquot of each day's urine collection was preserved with acid and refrigeration. These daily aliquots were mixed and sampled for nitrogen and energy determinations at the end of the experimental period. Analyses of feed and feces were made by the usual procedures. The urinary nitrogen was determined by the Kjeldahl method and the urine energy by a modification of methods used at the Illinois and Pennsylvania stations.

Metabolizable energy of the rations was calculated from data obtained as indicated in the preceding paragraph and by using the equation, $E=2.41X+9.80$, to calculate the methane produced by fermentation; see Swift et al. (*13*). Correction for nonmetabolizable portions of protein gain was also made.

TABLE 1.—*Composition of rations fed to lambs (air-dry basis)*

Ration No.	Dry matter	Protein	Fat	Fiber	Nitrogen-free extract	Ash	Energy
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Calories per kilogram*
1	89.7	12.3	3.09	13.7	57.7	2.9	4,081
2	91.5	12.5	2.60	15.5	58.2	2.7	4,101

Since a detailed description of the method used in determining the energy content of the urine was not found in the recent literature, a convenient technique is described.

The urine aliquot, preserved with acid, is neutralized with alkali. Fifteen ml. of this aliquot are placed in an evaporating dish and dried at 60° C. until a thick syrup is formed. Then with the aid of hot distilled water and a stirring rod the sample is transferred to a combustion cup containing a weighed amount of cellulose block. This material is redried and a second washing is made from the evaporating dish to assure complete transfer of the urine. At this time the block is distributed over the cup by shredding with a pointed glass rod. After a final drying the sample is ready for combustion in the calorimeter.

The gross energy content of the cellulose used must of course be determined by separate analyses and this value subtracted from the total heat evolved. Since some nitrogen will be lost in the course of drying, samples must be prepared in the same way as for the energy determination, and the loss of nitrogen by drying determined by the Kjeldahl method. If it is assumed that the nitrogen lost originated from urea, each gram of nitrogen lost will represent a loss of 5.424 calories. Under the conditions of the experiment this loss of energy in drying amounted to from 2 to 4 percent of the gross energy of the urine.

RESULTS AND DISCUSSION

Table 2 presents a summary of the data obtained. All differences observed between the two rations are statistically significant at the 1-percent level except those for fiber digestibility.

From the data given in table 2 it may be determined that the lambs on the supplemented ration digested 91 percent as much dry matter, 82 percent as much protein, 117 percent as much fat, 92 percent as much nitrogen-free extract, and 93 percent as much energy as on the alfalfa hay-corn ration. Sixteen percent of the digestible energy of the alfalfa hay-corn ration and 15 percent of that of the supplemented

TABLE 2.—*Percentage digestibility of rations*[1] *for lambs, and metabolizable energy expressed in calories per kilogram and as percent of the gross energy*

Lamb No.	Ration No.	Dry matter	Protein	Fat	Fiber	Nitrogen-free extract	Energy	Metabolizable energy	Energy
								Calories per kilogram	*Percentage of gross*
1	1	79.3	77.0	72.7	58.2	87.6	77.8	2,981	65.5
	2	72.4	61.7	87.1	52.2	81.3	71.7	2,788	60.9
5	1	77.8	72.8	74.0	56.3	86.5	76.3	2,933	64.3
	2	70.2	58.6	86.3	50.1	79.5	69.4	2,711	59.1
6	1	79.7	78.5	72.0	55.8	88.4	78.4	3,008	66.0
	2	72.0	67.3	85.2	56.0	77.7	71.0	2,760	60.3
7	1	76.5	73.4	68.2	53.8	84.9	74.6	2,850	62.6
	2	71.8	60.7	85.9	53.8	80.4	70.8	2,743	59.8
9	1	78.4	75.5	74.6	50.4	86.2	76.9	2,979	65.5
	2	72.1	60.5	78.9	51.2	81.9	70.7	2,744	59.8
Average	1	78.3	75.4	72.3	54.9	86.7	76.8	2,950	64.8
	2	71.7	61.8	84.7	52.7	80.2	70.7	2,749	60.0

[1] As explained in connection with reference to table 1, ration 1 was composed of 44.5 percent of good-quality chopped alfalfa and 55.5 percent of yellow corn. Ration 2 was composed of 50.6 percent of yellow corn, 15.5 percent of cottonseed meal, 4.0 percent of alfalfa leaf meal, and 29.9 percent of cottonseed hulls containing 10 percent of molasses.

hull ration were lost to the animals by way of urine and fermentation in the gastro-intestinal tract. Although of similar gross energy content, the supplemented hull ration contained only 93 percent as much metabolizable energy as the alfalfa hay-corn ration. This difference was due to the lower digestibility of the former ration.

In view of the similarity of composition of the rations, as judged by the usual feed analyses, it was evident that some factor other than those shown by analysis was affecting the digestibility of the rations. Lignin analyses, made by the 72 percent H_2SO_4 method as modified by Ellis, Matrone, and Maynard (*1*) gave a possible clue. The lignin content of the alfalfa hay-corn ration was 4.40 percent and of the supplemented hull ration 9.45 percent, on the dry basis. Previous studies at this station by Forbes and Garrigus (*6*) have shown a close correlation between lignin content and digestibility of pasture forages. The present investigation also indicates a possible influence of lignin on the digestibility of mixed rations.

The common explanation for the inhibiting effect of lignin on digestibility is that the lignin forms a mechanical barrier to the action of digestive juices. Such an explanation, however, does not explain all the observations of this study. For example, although a very minor portion of the total protein of the supplemented hull ration was contained in the highly lignified hulls, the lignin apparently lowered the total protein digestibility by 18 percent. The supplemented hull ration was more compact than the alfalfa hay-corn ration, although the hay in the latter ration was chopped into about 1-inch lengths. It is possible that rumination by animals receiving the more compact ration was less extensive. Such a finding has been made in studies with dairy heifers by Swanson and Ragsdale (*12*), although they found no difference in digestibility between chopped and finely ground hay. The compactness of the supplemented hull ration might be a factor in causing the difference in digestibility between the two rations. Another factor might be the presence in cottonseed hulls of some substance inhibiting the normal action of the rumen microflora or the digestive enzymes. Wise (*14*), in summarizing the action of bacteria on lignin, notes that isolated lignin is sometime inhibitory to bacterial growth. However, a similar effect of lignin "in situ" has not been proven and specific data on this subject, must await further investigation.

The data here presented also afford evidence that under some circumstances the material analyzed as lignin by the method used in this study may be digested appreciably by ruminants. Ninety-seven percent of the lignin fed in the alfalfa hay-corn ration was recovered as compared with 85.7 percent when the supplemented hull ration was fed. It is possible that some of the resins and pentosans present in cottonseed hulls are not affected by the reagents used in the lignin analysis but are subject to some degree of change in the digestive tract of the sheep.

The average daily nitrogen balance of the lambs on the alfalfa hay-corn ration was 2.86 grams (range 1.51 to 4.03) and that on the supplemented hull ration 2.59 grams (range 0.57 to 3.74). These values are not significantly different.

Carotene analyses were not made but calculations based on average published analyses showed that the rations might be expected to contain an adequate amount of this nutrient. The supplemented hull ration contained only 70 percent of the recommended allowance of calcium. This deficiency would not be likely to affect the results of an experiment of this type.

In view of the desirability of expressing the nutritive value of rations in terms of metabolizable energy, it is of interest to compare the results obtained in this experiment with those calculated from digestible nutrients by the method of Axelsson, cited by Forbes and Thacker (*5*). In this computation the following factors were used per gram of digestible nutrient: Protein 4.4, fat 8.0, fiber 2.9, nitrogen-free extract 3.7. Table 3 shows the estimates of metabolizable energy obtained by the method of Axelsson (calculated) and in this experiment (experimental).

Axelsson's factors were derived for use in determining the metabolizable energy of rations for cattle, but the data presented herewith show that these same factors may also be useful with rations for sheep.

TABLE 3.—*Comparison of methods of calculating metabolizable energy of rations for sheep*

Lamb No.	Metabolizable energy (calories per kilogram of feed)			
	Alfalfa hay-corn ration		Supplemented hulls ration	
	Calculated	Experimental	Calculated	Experimental
1	3,006	2,981	2,738	2,788
5	2,948	3,008	2,663	2,711
6	3,019	2,850	2,700	2,760
7	2,887	2,979	2,713	2,743
9	2,940	2,950	2,719	2,744
Average	2,960	2,950	2,707	2,749

SUMMARY

The digestibility and metabolizability of two rations by sheep are reported. Ration 1 was composed of alfalfa hay and corn, while ration 2, similar in gross nutrient analysis, was composed of corn, cottonseed meal, alfalfa leaf meal, cottonseed hulls, and molasses.

The lambs fed ration 2 digested 91 percent as much dry matter, 82 percent as much protein, 117 percent as much fat, 92 percent as much nitrogen-free extract, and 93 percent as much energy as did those fed ration 1. Of the digestible energy of both rations 85 percent was metabolizable.

The metabolizable energy of the rations as calculated from this experiment was in close agreement with that calculated by the method of Axelsson.

A convenient method for determining the gross energy value of the urine is described.

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STABILITY OF CAROTENOIDS IN GROUND DEHYDRATED CARROTS [1]

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INTRODUCTION

The possibility that antioxidants might protect the carotene pigments in dehydrated carrots was suggested by the observation (Weier, 8) that these pigments dissolved in intracellular oil droplets when small pieces of carrot root were dried. Upon storage the pigments disappeared from the oil droplets whereupon they gave a positive test with Schiff's reagent, indicating the occurrence of oxidative rancidity in the oil. It seemed possible that the addition of an antioxidant to the oil droplets would retard pigment degradation in dehydrated carrots.

Since in blanched, but undried carrot dice, as much as 90 percent of the pigment may disappear in 24 hours, the first tests were carried out on this type of material (*9*, *10*).[2] Positive results were obtained: ascorbic acid, nordihydroguaiaretic acid, oxalic acid, and phosphate buffer at neutrality greatly retarded pigment breakdown. That antioxidants will also protect the pigment in dehydrated carrots has been shown in some preliminary tests (*11*).

Lovern (*4*) and his associates studied the influence of antioxidants on the shelf life of dehydrated carrots. They found that under certain conditions, the pigment in ground dehydrated carrots received a slight amount of protection while that in dehydrated dice did not.

The problem of protecting the carotenoid pigments in dried carrots is complicated by a number of physical factors that are not present in experiments on pure pigments in pure solvents. Some of these factors are the following:

(1) The concentration of the pigment and the nature of the oil in which the carotenoids are dissolved is not known.

(2) The particulate nature of the oil-pigment complex presents an enormous surface which is unquestionably a factor in the oxidation of the pigment. Both the area and chemical nature of this surface are of importance.

[1] Received for publication August 30, 1948. This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number *203* in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army. The work was done at Davis, Calif.

[2] Italic numbers in parentheses refer to Literature Cited, p. 501.

(3) The cellular structure of the carrot root, varying in different regions of the root, must influence the rate of diffusion of the oxygen and consequently the rate of oxidation of oil and pigment.

(4) Carbohydrate content, particularly starch, which becomes gelatinized during blanching, may be a factor in limiting the rate of pigment degradation.

(5) The total amount of pigment present in the dried dice is likely to influence the rate of breakdown. This factor may not be, however, directly related to the mass action law. The concentration of the pigment in the oil droplets may be uniform but there may be variations in the number of oil droplets per unit volume. This would result in a great increase in surface with an increased rate of pigment breakdown.

6) Natural antioxidants such as tocopherols are known to be present in carrots and they appear to have effect in protecting the pigment when it is concentrated in a natural oil extract. The relationship of such natural antioxidants and the oil and pigments in raw and dehydrated carrots is not known.

(7) Season of harvest and preprocessing handling may influence the stability of the pigment.

Although the main interest of these investigations as indicated was the eventual testing of the influence of certain antioxidants on the rate of breakdown of the carotenoid pigments, certain details regarding the breakdown of the pigments in blanched dehydrated carrots under accelerated storage must first be ascertained. This paper discusses the variation in the pigment concentration in carrot roots during growth and the localization of the pigment within the root. Rates of breakdown in dehydrated carrots under accelerated storage conditions are reported for the total pigment complex, for alpha and beta carotenes, and for the more strongly adsorbed carotenoid components. The influence of temperature and oxygen concentration on the rate of pigment degradation is also discussed.

MATERIAL AND METHODS

PREPARATION AND STORAGE

Imperator carrots (Ferry-Morse seed) were used in these studies. Unless otherwise stated all carrots used were grown in Davis and harvested immediately before needed.

Diced carrots were prepared for the storage experiments by blanching in steam for 5 minutes, after which they were dehydrated at 140° F. for 8 to 10 hours to 6 to 7 percent moisture, by the 40-hour vacuum oven method, in an experimental dehydrator built by the Agricultural Engineering Division of the University of California. After drying, the dice to be used in the accelerated storage tests were ground to pass a 20-mesh sieve. In order to insure uniform particle surface, the fine particles were sifted through a 48-mesh sieve and discarded. Five-gram samples of these ground carrots were stored in stoppered 300-ml. Erlenmeyer flasks which were opened at regular intervals. When larger amounts of ground carrots were stored, unless otherwise stated, atmospheric oxygen was available to the sample.

PIGMENT DETERMINATION

For total pigment determinations 0.1-gram aliquots of the dehydrated material were rehydrated by pouring boiling water over them and allowing them to stand for about 30 minutes. The water was decanted off and the pigments were completely extracted in acetone and made up to 100-ml. volume. The optical density of this solution was measured on a Lumetron Colorimeter using a 440-mμ filter. Since in the majority of these tests the pigments were not separated it seemed unwise to express the results as milligrams of beta carotene.

All extracts were handled in low actinic red glassware and when measurements were not taken immediately after preparation of the extract it was kept at 4° C. All measurements were made within 24 hours of the preparation of the extract.

In connection with storage and antioxidant investigations of dehydrated carrots, it is convenient to use carotenoid degradation rates in determining deterioration of quality. In its simplest form such a procedure involves the extraction of the total pigment with acetone and the determination of the total optical density of this crude extract with a photoelectric colorimeter, as just described. Carrots, however, contain a series of polyene compounds. Consequently, in a detailed investigation of storage changes it is important to ascertain the relative stability of at least the major members of this series and to determine whether there is any selective antioxidant effect upon them.

The approach to this aspect of the problem was divided into two parts, (*1*) a study of pigment changes with growth of the carrots, and (*2*), a study of the stability of individual pigment components in dehydrated carrots under accelerated conditions of storage and subject to antioxidant treatments.

Samples were taken for chromatographic analyses from the regularly prepared and stored material. One-gram portions were rehydrated, drained, extracted with acetone, transferred to petroleum ether and washed three times with water. They were then dried with anhydrous sodium sulphate and evaporated under vacuum at room temperature in the dark to about 1- to 2-ml. volume in most cases.

The chromatographs were prepared on 1:1 magnesia [3] Super-Cel columns (13-mm. diameter) using petroleum ether as a pigment solvent. Petroleum ether with 4 percent acetone was used for the development. About three-fourths of an inch of anhydrous sodium sulphate was placed at the top of the column to insure complete removal of all moisture before development.

The columns were protected from the light and adequate precautions were taken to prevent drying. After the development the individual components were either collected as liquid fractions below the column or the column was pushed out and immediately divided and the individual bands placed in acetone. In the latter case (the usual procedure) the adsorbent was removed from the pigment fractions by filtering through fine sintered glass filters and washing with acetone. Thus optically clear solutions of the individual fractions were obtained.

[3] Adsorptive powdered magnesia No. 2641, Westvaco Chlorine Products, Newark, Calif.

In general only three groups of pigments were separated: (1) Alpha carotene, (2) beta carotene, (3) the balance of the carotenoids called group "A" for convenience. In some runs zeta carotene (which is adsorbed just above beta carotene) was also determined. After preparation and proper dilution of the samples the absorption of light by each was measured, using a photoelectric colorimeter and a 400-mμ filter. The results are reported as relative optical density calculated on the basis of the pigment in 1 gram of dehydrated carrot dissolved in 100 ml. of solution.

Losses in transferring to petroleum ether and in chromatographing were determined and recovery was usually more than 90 percent based on photoelectric determinations using the 440-mμ filter. Table 1 shows a typical set of analyses with losses in total pigment incurred in the process.

Losses in individual fractions were also checked to see whether there were significantly greater losses in one fraction than in another. The alpha and beta carotene fractions were rechromatographed to determine whether the percentage lost by either fraction was significantly greater than the total loss for the combined fractions. Table 2 shows that in both cases approximately the same percentage of loss was obtained as when total pigment was used.

TABLE 1.—*Percentage recovery of total carotenoid pigment after chromatographing from sample of blanched, dehydrated, ground carrots stored at 40° C.*

[Figures are optical density determinations made with a photoelectric colorimeter using a 440-mμ filter]

Item measured	July 25	July 29	Aug. 1	Aug. 5	Aug. 8
Optical density before chromatographing	3.645	3.630	3.655	3.380	3.080
Optical density after chromatographing	3.422	3.512	3.367	3.190	2 960
Percent recovered	93.8	96.8	92.2	93.4	96.0

TABLE 2.—*Losses in individual carotenoid pigments during rechromatographing*

[Figures are in relative optical densities determined using a 440-mμ filter and a photoelectric colorimeter]

Fraction	Before rechromatographing	After rechromatographing	Percent lost in rechromatographing
Oct. 19:			
Alpha carotene	1.487	1.399	5.9
Beta carotene	2.617	2.438	6.8
Nov. 28:			
Alpha carotene	.347	.313	9.8
Beta carotene	.335	.310	7.5
Group "A"	.177	.165	7.0

PIGMENT CHANGES WITH GROWTH OF CARROTS

It has been reported (*5*) that there is no marked variation in the ratio of alpha to beta carotene with growth of carrots during a period of from 9 to 26 weeks after germination, although the total carotene content increased about 25 times during this period. A further test of this point, particularly during the early stage of growth, was made in the present study. Changing concentrations in the various carotenoid fractions from the third to the ninth week of carrot growth are

shown in figure 1. It should be noted that the results reported are optical densities obtained using a 440-mμ filter and are not absolute values of carotenoid concentration.

Because of the lack of complete uniformity of germination and growth the carotenoid concentrations per unit fresh weight of carrot are plotted against the average weight of individual carrots rather than age. The last points on the graph show the pigment content of 9-week-old carrots whose average weight was 10.8 gm. per carrot. Carrots of uniform size were selected for each set of determinations. The first determinations were made when the seedlings were from 2 to 8 cm. in length and averaged 0.066 gm. fresh weight (90 percent water). Two hundred and fifty-nine carrots were used for the analysis. The last determinations were made on carrots 15 cm. long and 10.8 gm. in weight. Only seven of these carrots were used in the analysis.

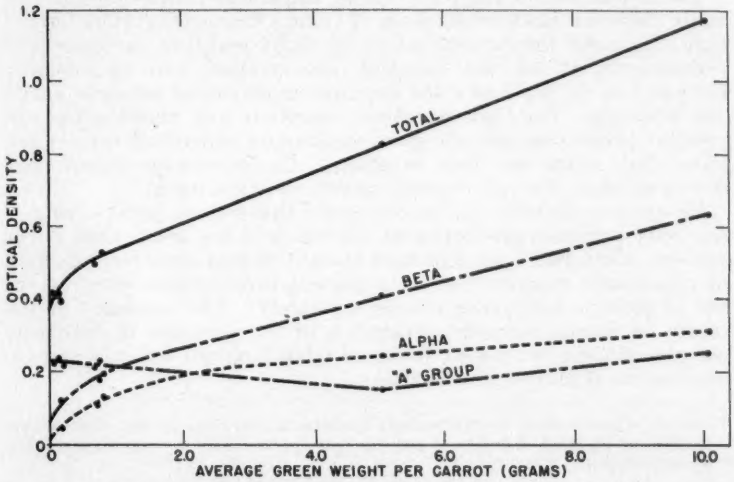

FIGURE 1.—Changes in carotenoid pigments in carrot roots during growth. Densities based on 10 gm. of fresh carrot per 100 ml. of solution. (Group "A" includes all carotenoid pigments more strongly adsorbed than beta carotene.)

The results show that the very young seedlings contain less alpha and beta carotene than the remainder of the carotenoid pigments—chiefly strongly adsorbed carotenols. During the development of the carrot the concentration of the carotenols within the tissue remains relatively constant while there is a uniformly rapid increase in alpha and beta carotene. There is some indication that beta carotene is present in a slightly greater proportion in the young carrots than in the older ones, i. e., the ratio of beta to alpha carotene dropped from 3.5 to 1.6 and then increased to slightly over 2 which was about the ratio found in the mature carrots of these plantings. The ratio of the beta and alpha carotene to the balance of pigments increased from 0.61 in the first sample to 3.8 in the 9-week-old carrots. In mature carrots of this strain this ratio is between 8 and 9.

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These data would indicate that oxidized carotenes, or perhaps colorless polyenes which were not determined, are the major polyene components in carrot seed, and that the alpha and beta carotenes are formed chiefly upon germination and growth of the seedling, being laid down in the manner of a waste product, and are at no time drawn upon to any appreciable extent as a reserve. On the other hand, the carotenols appear to be of fairly uniform concentration during the development of the carrots and presumably occur in high concentration in the seed. This is an agreement with conditions found by Strain (*7*) for barley seeds. In this latter case the principal constituents of seeds were the xanthophylls, carotenols with large amounts of fluorescent colorless materials and small amounts of beta carotene being present. Unfortunately, limitations of facilities prevented the determination of the colorless polyenes such as phytofluene.

The population of the plantings of Imperator carrots used in this study showed a fairly wide range of color. Chromatographs showed that in general the concentrations of alpha and beta carotenes and consequently of the total pigment concentration were considerably increased as the depth of color increased in carrots of the same age in any planting. The lightest colored carrots in any planting had the greatest proportion and the greatest absolute amount of carotenoids other than alpha and beta carotenes. In the storage experiments described later, the light-colored carrots were not used.

Harper and Zscheile (*2*) have reported that several varieties of carrots have a greater proportion of carotenols in the xylem than in the phloem, which had a predominant amount of beta carotene. Certain of the storage experiments in the present investigation involved the use of phloem and xylem tissues separately. The summary of the results of chromatographic separation of the pigments in composite samples of these two issues, shown in table 3, agrees with the general conclusions of Harper and Zscheile.

TABLE 3.—*Contribution by the various carotenoid fractions to the total optical density of pigment extracts of phloem and xylem tissue in imperator winter-grown carrots*

Sample No.	Percent of total optical density			Total optical density
	Alpha carotene	Beta carotene	"A" group	
1.—Phloem	19.4	73.3	7.3	2.970
2.—Phloem	19.0	73.3	7.7	3.210
3.—Phloem	21.1	72.1	6.8	2.970
4.—Xylem	14.4	66.0	19.6	1.137
5.—Xylem	16.4	67.2	16.4	1.092

RATE OF PIGMENT BREAKDOWN UNDER ACCELERATED STORAGE

STORAGE IN AIR

TOTAL PIGMENT DEGRADATION

The dissolution of the carotenes in the oil droplets and the positive Schiff tests given by these droplets after the degradation of the pigment, suggested that the rate of pigment breakdown might follow a

typical fat-oxidation curve, and that an initial induction period, followed by a rapid rate of oxidation might be expected. With but few exceptions this type of breakdown was observed: There was an induction period of from 3 to 5 days followed by a period of increased rate of pigment breakdown, which was generally maintained until from 50 percent to 60 percent of the pigment was degraded. The points along this portion of the curve, in all experiments, fell on a straight line when plotted on standard coordinate paper (fig. 2). Regression lines were run for this portion of the curve for many experiments. The fit was unusually good, the standard error being between 2 and 4 percent.

After the decomposition of 50 percent to 60 percent of the pigment, the rate of breakdown slowed greatly. This occurred in many experiments, regardless of treatment, in such a manner that the residual pigment content after long storage times—from 30 to 40 days in experiments with ground carrots—was approximately the same.

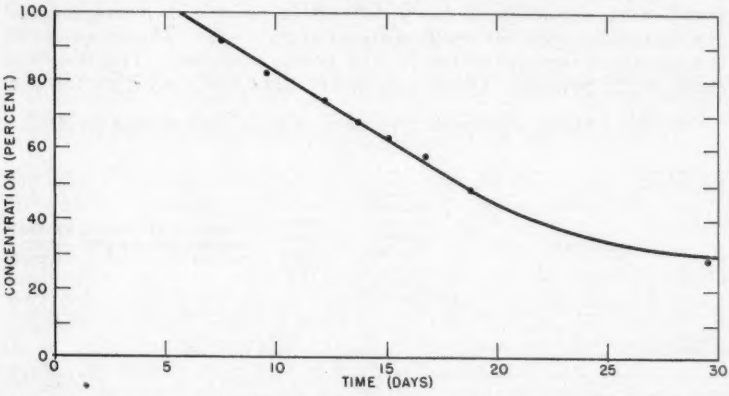

FIGURE 2.—Rate of carotenoid degradation in blanched, dehydrated, ground carrots stored in air at 40° C.

Since the main interest was to extend the induction period and to greatly retard the rate of breakdown during the straight-line portion of the process no attention was paid to the latter stages of breakdown.

Because of the shape of the breakdown curve it is of importance, when making comparisons between various experiments, to be certain that the same portions of the curve are being compared.

The series of experiments herein reported extended over a period of 2 years. In analyzing the data obtained it was necessary to know the extent of the variations (1) in tests run at the same time on separate lots of carrots from one or more plantings, and (2) in tests run at different times on lots of carrots from different plantings.

In order to test the first point (the second will be discussed in a subsequent section) six series of tests were run on each of two plantings of carrots. The roots in one planting were 6 months of age, those in the second 3 months old. The results shown in figure 3 are those obtained from the younger roots. The older roots yielded figures

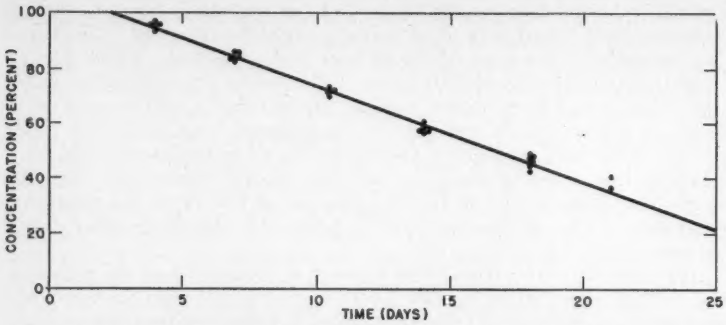

FIGURE 3.—Rate of carotenoid degradation in six samples of blanched dehydrated ground carrots stored in air at 40° C.

which were so similar as to be difficult to include in a single small graph together with the result of the younger roots. The curve shown is a standard regression line for the points involved. The standard error is 2.2 percent. Other significant data are shown in table 4.

TABLE 4.—*Typical carotenoid breakdown in ground dehydrated carrots stored at 40° C.*

Age of roots	Average carotenoid content in gamma of Eastman carotene per gram of dried carrots	Initial optical density reading	Percent breakdown per day	Percent breakdown 20 days	Standard error (percent)
3 months	710	0.240	3.11	55	2.2
6 months	1,260	.420	3.49	57	3.2

The stored samples of ground dehydrated carrots gradually lost weight during storage. A 5-gram sample of ground dried carrots under the storage conditions described lost approximately 0.26 gm. (5 percent) in 40 days' storage. During 20 days' storage, the normal duration of the majority of experiments, the weight lost was 0.07 gm. Carotenoid concentrations calculated for the 20-day storage period should therefore be decreased slightly. The error is small and reasonably uniform in all experiments. No corrections for it have been made.

DEGRADATION OF INDIVIDUAL PIGMENTS

The characteristic curve representing pigment degradation in blanched ground carrots stored at 40° C. in air-tight containers is shown in figure 4. In this case no induction period was observed. This graph shows the contribution of alpha and beta carotenes and the more strongly adsorbed carotenoids to the total pigment breakdown curve. It should be emphasized again that the results represent only the optical density determined by use of the photoelectric colorimeter using the 440-mμ filter and should not be interpreted as representing

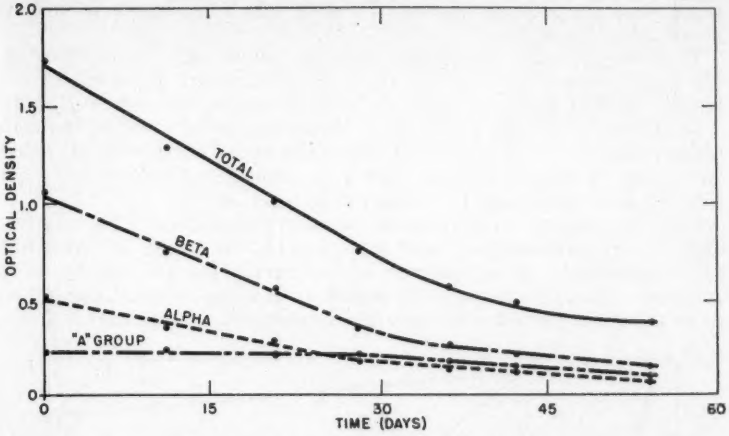

FIGURE 4.—Rate of degration of individual carotenoid pigments during storage of blanched, dehydrated, ground carrots at 40° C. in air. Optical densities based on 1 gram of dehydrated carrots per 200 ml. of solution. (Group "A" refers to all carotenoids more strongly adsorbed than beta carotene).

the actual concentration of the various pigment fractions. However, the values do represent the contribution of the individual components to the total optical density determined with the colorimeter at this wave length. It will be noted that the alpha and beta carotene fractions both have curves of the same general shape, and because they constitute the major portions of the pigment present, the total pigment degradation curve is of the same shape. Mackinney and Fratzke (*6*) have also observed that alpha and beta carotenes oxidized at about the same rate during storage of dehydrated carrots.

The more strongly adsorbed pigments (zeta, gamma carotenes, and the carotenols) show a fairly constant density throughout the storage period with a slow decrease appearing only when the rate of breakdown of alpha and beta carotene began to level off. This might indicate that degradation products of alpha and beta carotenes contribute to the density of the more strongly adsorbed group. A further indication that this is the case may be found in figure 6 which shows a small but definite increase in this group during the early storage period when there was a rapid breakdown of alpha and beta carotenes. This increase has been observed in a number of experiments.

GAS STORAGE

Hoffman, Lum, and Pitman (*3*) have studied the relation between rate of carotene degradation in dehydrated alfalfa and oxygen tension. They concluded "that at least the initial rates of carotene destruction are very nearly proportional to the percentages of oxygen in the storage." When oxygen tension varied slightly or not at all, i. e., when the gas mixture containing 3 percent oxygen was renewed daily, the rate of carotene breakdown was directly proportional to time. In their experiments the dried alfalfa meal occupied approximately 60

percent of the storage container, which was a Schwartz U-tube of about 26-cc. capacity.

In the experiments with ground dehydrated carrots, herein reported, the 5-gm. sample in a 300-ml. flask occupied about 1 percent of the volume of the container. The air in the container was renewed every 3 or 4 days. Although gas analysis of the atmosphere within the containers was not made the conditions of the experiment were such that variations of oxygen tension within the storage containers were not a factor in determining the course of the reaction.

Only one series of comparisons between O_2, air, and CO_2 was run (fig. 5). Some breakdown does occur in CO_2, the nature of which was not determined. A comparison of the curves for air and O_2 is of interest. The curve for rate of breakdown in air is, as usual, comprised of an induction period plus a straight line portion of rapid breakdown.

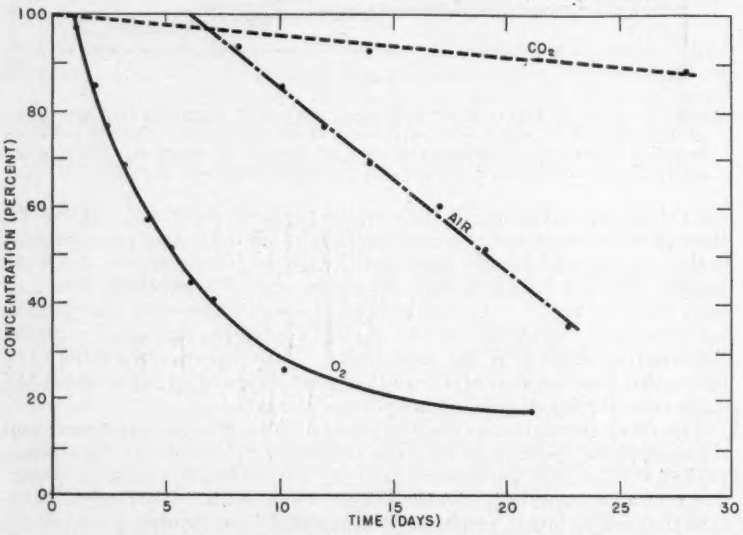

FIGURE 5.—Influence of storage atmosphere on carotenoid degradation in blanched, dehydrated, ground carrots stored at 40° C.

In oxygen the induction period is shorter and a true straight line period of breakdown appears to be absent. Instead the points lie along an exponential curve. When these points are plotted on semilog paper a straight line results. This indicates that the lower oxygen tension of the air is a modifying factor in the pigment breakdown. With a great excess of oxygen the points obtained suggest that the rate of pigment degradation is more closely proportional to the amount of pigment present.

In spite of the straight line obtained for breakdown in air, pigment concentration, at least at the start of the experiment, influences the rate of pigment breakdown.

A further study in which pigment breakdown was accelerated by storing the ground carrots in an atmosphere of oxygen showed that

alpha and beta carotenes under these conditions undergo oxidative decomposition at about the same rate. The ratio of beta to alpha carotene was 3.61 at the start of the experiment, increased to 4.14 at 5 days, and was 3.11 at the end of 27 days when 90 percent of the pigment was destroyed. At the same time there was, however, a slight increase in the density of the strongly adsorbed pigments followed by a decrease (fig. 6).

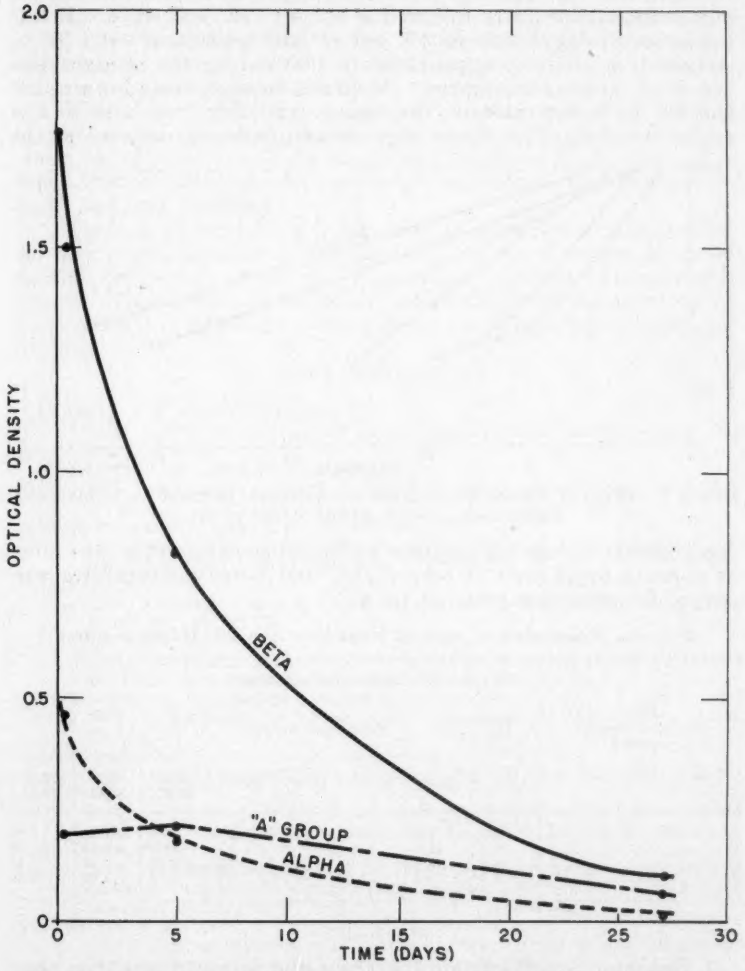

FIGURE 6.—Carotenoid degradation in blanched, dehydrated, ground carrots stored at 40° C. in oxygen. (Group "A" refers to all carotenoids more strongly adsorbed than beta carotene.) Densities based on 1 gram of dehydrated carrots per 200 ml. of solution.

EFFECT OF TEMPERATURE

One difficulty encountered in using accelerated breakdown tests is that of establishing that the accelerated reaction is following a course that is similar to the slower reaction, about which information is desired. It has, for instance, been shown that rapid fat oxidation at elevated temperatures is not strictly comparable to oxidation at lower temperatures (*1*). In order to determine the best temperature for studies on carotene degradation in samples of dehydrated carrots, pigment breakdown was followed at 30°, 40°, 50° and 60° C. It was found that the Q_{10} [4] between 30° and 40° and between 40° and 50° C. remained constant at approximately 1.60 during the straight line period of carotene breakdown. The ratio between breakdown at 50° and 60° C. is not constant, decreasing gradually from 1.48 to 1.24 (table 5 and fig. 7). Forty degrees centigrade was selected as the

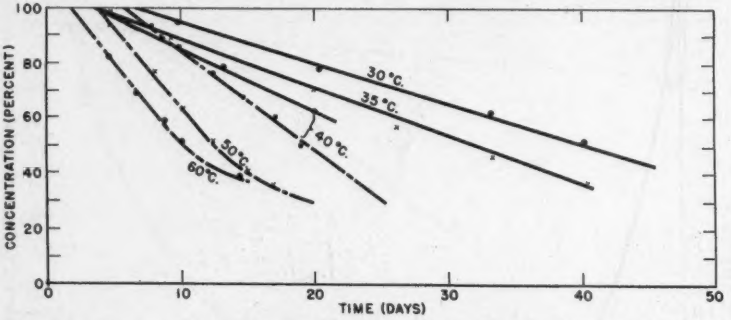

FIGURE 7.—Effect of storage temperature on carotenoid degradation in blanched, dehydrated, ground carrots stored in air.

experimental storage temperature with good assurance that the course of pigment breakdown at both higher and lower temperatures was comparable with that found at 40° C.

TABLE 5.—*Comparison of pigment breakdown at different temperatures*

Percent of pigment degraded	Time in days for pigment degradation in—					Q_{10}		
	Experiment No. 1, at—		Experiment No. 2, at—					
	30° C.	40° C.	40° C.	50° C.	60° C.	30 to 40° C.	40 to 50° C.	50 to 60° C.
10	4.0	7.8	8.4	5.5	3.7	1.80	1.52	1.48
20	20.2	12.0	11.0	7.1	5.3	1.67	1.60	1.33
30	26.8	16.2	13.8	8.7	6.7	1.65	1.58	1.29
40	35.2	20.6	16.5	10.3	8.3	1.65	1.60	1.24

SUMMARY

1. Seedling carrots contain less alpha and beta carotene than they do carotenols and other strongly adsorbed carotenoids. During the

[4] The temperature coefficient (Q_{10}) of a process is defined as the number of times that the rate of the process increases with 10° C. rise in temperature.

development of the carrots the concentration of carotenols per unit fresh weight remains fairly constant but there is a steady increase in amounts of alpha and beta carotenes.

2. Lighter portions of carrot roots have a higher absolute value of the more strongly adsorbed pigments (zeta, gamma, and the carotenols) than do the more highly colored portions of the roots. There is a higher proportion of these strongly adsorbed pigments in the xylem. The phloem has a high proportion of beta carotene.

3. Carotene breakdown in air in dehydrated ground carrots stored at 40° C. showed an initial period of no degradation or very slow degradation, a second period of rapid degradation, and a third or final period when pigment breakdown was very slow.

4. The rate of breakdown of alpha and beta carotene was similar to that of the total pigment complex. The more strongly adsorbed pigments (zeta, gamma, and the carotenols) remained fairly constant in concentration, showing a decrease only when the rate of breakdown of alpha and beta decreases.

5. The rate of breakdown in air during the second or rapid period, for any given experiment was constant. In pure oxygen an exponential curve was obtained. Apparently pigment breakdown in ground dried carrots in air is not proportional to pigment concentration but it is related to concentration when the sample is stored in oxygen.

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THE INFLUENCE OF SELECTED ANTIOXIDANTS ON DEHYDRATED CARROTS [1]

By T. E. WEIER, *associate botanist*, and C. R. STOCKING, *assistant botanist, Division of Botany, California Agricultural Experiment Station.*

INTRODUCTION

Certain external factors influencing the rate of carotenoid degradation in dehydrated carrots have been considered in a companion paper. This paper reports: (1) The influence of certain internal factors such as carotene and starch content on pigment degradation; and (2) the ability of certain selected antioxidants to retard the rate of pigment degradation.

MATERIAL AND METHODS

The source of carrots and methods of pigment analysis have been reported by Stocking and Weier (*6*).[2]

Antioxidants were generally applied by soaking the blanched dice in solutions of selected chemicals. Except for sodium metabisulphite no effort was made to determine the amount of antioxidant within the dehydrated dice. While this is an important aspect of the problem the major interest was first to determine whether or not the carotene could be protected.

In the majority of the experiments herein reported the SO_2 content was approximately 1,000 parts per million. This concentration of SO_2 in the dehydrated dice was obtained by soaking the blanched dice in a 0.12-percent solution of $Na_2S_2O_5$.

Unless otherwise indicated the samples for the accelerated breakdown tests were ground, after dehydration, to pass a 20-mesh screen and be retained on a 48-mesh screen. They were stored in air at 40° C.

The problem was to determine whether or not the pigment in dehydrated carrots could be protected by antioxidants and if so which antioxidants were of highest value. All experiments were conducted in duplicate, unpromising antioxidants were immediately discarded, others were tested a second time, and the best (such as pyrogallol) were tested five or six times or more under varying conditions. Pyrogallol was tested 10 times over a 2-year period.

[1] Received for publication August 30, 1948. This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number *204* in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army. The work was done at Davis, Calif.

[2] Italic numbers in parentheses refer to literature cited, p. 515.

RATE OF PIGMENT BREAK-DOWN IN GROUND SAMPLES UNDER ACCELERATED STORAGE CONDITIONS

VARIATION IN TOTAL PIGMENT CONTENT

Previous reports on the pigment content of carrot roots indicate that the pigment increases to a maximum at from 16 to 22 weeks after planting; thereafter the pigment remains constant, (1), (3). Our own results confirm these experiments. The pigment increases rapidly during the active growing season, after which it remains practically constant. This applies to carrots grown during both summer and winter seasons. Roots resulting from September sowings did not accumulate so much pigment as those planted in March. Hansen (*3*) reports similar results with carrots grown in Oregon.

Cessation of pigment deposition, in general, seems to be correlated with a natural decrease in the growth rate. In Oregon and New York this coincided with the onset of fall weather; in the University of California's garden at Davis, deposition of pigment ceased in early September, with a month of warm weather remaining. Pigment deposition was halted in the spring by bolting. The relationships between deposition of pigment and season of the year is shown in figure 1.

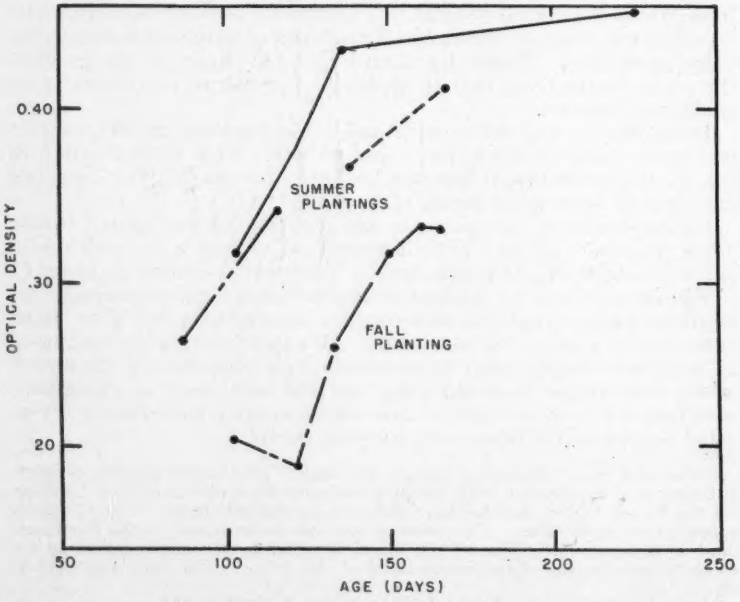

FIGURE 1.—Natural increase in carotenoid content in carrot roots harvested at various ages and at different seasons. Optical density is based on a sample of one-tenth of a gram of dehydrated carrots to 100 milliliters of solution.

This natural variation in pigment content increases the difficulty of accurately comparing the rates of pigment break-down in dehydrated carrots. The extreme variation, occurring between young and old summer-grown roots may be as much as 200 percent.

During the course of the investigation 24 separate experiments in which the diced carrots received no treatment other than blanching and drying were carried out. These experiments included carrots of all ages and of varying total pigment contents. They cover three growing seasons; the summer of 1945, the fall and spring of 1945–46, and the summer of 1946.

A comparison of the results of these experiments showed that as the initial pigment content increased the percentage of pigment remaining after a 20-day storage period also increased. This means that comparisons between different experiments based on percentages may lead to erroneous conclusions, in that a larger proportion of pigment remaining after a stated time interval may be caused by a larger initial pigment content rather by a variation in treatment. For the same reason a direct comparison between the absolute amount of pigment decomposed or of pigment remaining after a stated period of time may lead to erroneous conclusions.

When the logarithm of the amount of pigment oxidized in 20 days is plotted against the initial pigment concentration a fairly direct relationship is observed. An increase in the initial pigment content results in a proportional increase in the amount of pigment oxidized. The agreement between different experiments is, however, not as close as would be expected under more ideal conditions, such as known concentrations of pure carotene in a pure oil (Bickhoff and Williams *2*).

The results of these 24 experiments are shown in figure 2. The individual points represent the log of pigment oxidized in 20 days (optical density of 100-milliliter acetone extract of 0.100 gram of ground carrots), in a given experiment, plotted against initial pigment content.

A regression line was run for these 24 points. It is the center broken line in figure 2. The significance of this line is at the 2 percent level and the standard error is $\pm .013$. The lined area on either side of the regression line represents this standard error. It may be assumed, therefore, that if the plot of the log of the pigment oxidized in 20 days against the initial pigment content, obtained in any experimental treatment, falls within this lined area the rate of pigment break-down is that which might be expected for a blanch-only experiment. If a particular treatment results in a rate of break-down which when plotted falls above this lined area, this treatment may be considered as accelerating the rate of break-down. If, on the other hand, the results of a given treatment fall below the lined area it may be assumed that that treatment is affording more protection to the pigment than blanching alone.

It must be remembered that there are two aspects to the rate of pigment break-down as measured by the amount of pigment oxidized during 20 days: First, the length of the induction period and, second, the rate of break-down during the straight-line period. In general,

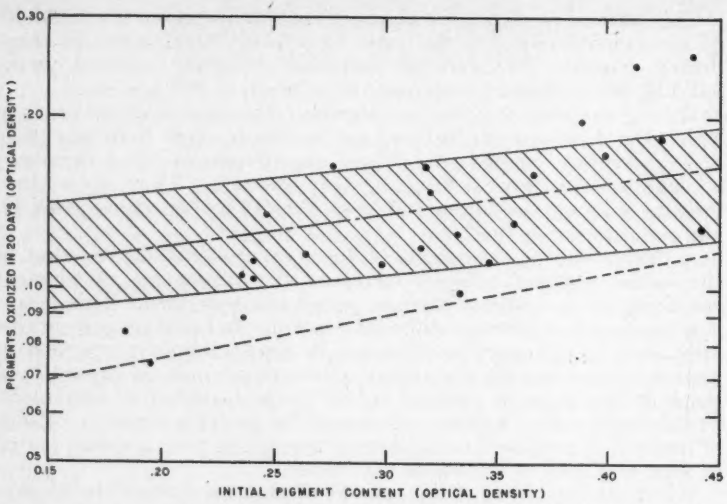

FIGURE 2.—A comparison of amount of initial pigment content (represented by optical density) with amount of pigment oxidized in 20 days (also represented by optical density) in dehydrated ground carrot samples stored in air at a temperature of 40° C. Lined area indicates range of variation in pigment oxidized in 20 days; broken line in center of lined area is the regression line. Results obtained in 24 blanch-only experiments.

it has been found that a lengthened induction period results in a slower rate of break-down during the straight-line period.

From a practical viewpoint any treatment to be of value must protect the pigment to an extent which is greater than the best protection afforded by blanching. Thus in the authors' system of comparison the results of a treatment to be considered good must fall on the graph in figure 2 below the lower evenly broken line, which indicates the upper limit of protection afforded by simple blanching.

THE EFFECT OF STARCH CONTENT ON CAROTENE STABILITY

If diffusion of oxygen into the tissue controls the rate of pigment breakdown under certain conditions, it seems logical that starch gelatinization may protect the tissue from this break down as Reeve (*5*) has suggested. Mann and Weier (*4*) pointed out the great variability in the starch content of individual carrots of a single strain. In order to test whether such variation in starch content would materially affect pigment break-down carrots were tested for starch and separated into high and low groups on the basis of a rough quantitative determination with iodine solution.

After their separation into high and low starch, the roots were split and the xylem separated from the phloem so that a series of four types was obtained: High-starch xylem, low-starch xylem, high-starch

phloem, and low-starch phloem. The amount of pigment broken down in 20 days was compared in each case with the normal blanch curve. Although the high-starch xylem and high-starch phloem both showed some less break-down of carotene than that in the low-starch tissue, the results were not sufficiently different to warrant further investigation.

PREPROCESSING STORAGE

Commercial processing procedures are often delayed beyond harvest time, necessitating certain post-harvest storage. A series of experiments was conducted to test the possibility that such storage might affect the rate of subsequent pigment degradation. Carrots were harvested and stored under the conditions indicated in table 1 and were then processed. When the amount of pigment broken down in them in 20 days was compared with the amount of pigment in the normal untreated blanched carrots no significant differences were observed in the rate of carotene break-down.

INFLUENCE OF SELECTED ANTIOXIDANTS

GROUND SAMPLES

As already indicated, Weier (*7*) and Weier and Stocking (*8*) have shown that under certain conditions the pigments in blanched and in blanched and dehydrated carrots could be protected to a certain extent by selected antioxidants. In our own laboratory 3-year-old samples of antioxidant-treated carrots still show a brilliant carrot color, whereas the color of comparable blanched-only and blanched—$Na_2S_2O_5$—samples are greatly faded.

TABLE 1.—*Comparison of amount of initial pigment and pigment oxidized in 20 days in carrots grown in the greenhouse and in the field, when stored after being harvested*

Postharvest storage conditions	Initial pigment content	Pigment oxidized in 20 days	Postharvest storage conditions	Initial pigment content	Pigment oxidized in 20 days
	(*Optical density*)	(*Optical density*)		(*Optical density*)	(*Optical density*)
Carrots grown in pots in greenhouse:			Carrots grown in field—Con.		
Control	0.325	0.117	32° F., 6 days, topped	0.274	0.132
32° F., 5 days	.352	.127	0° F., 6 days, topped	.340	.109
0° F., 5 days	.372	.134	Control	.311	.123
Carrots grown in field:			70° F., 4 days, topped	.282	.116
Control	.330	.099	70° F., 4 days, not topped	.289	.125
95° F., 6 days, topped	.281	.101	40° F., 4 days, topped	.311	.146
65° F., 6 days, topped	.261	.081	40° F., 4 days, not topped	.320	.154

Previous reports of the action of antioxidants have given only the percentage of pigment remaining in the stored sample after relatively short periods of storage. In table 2 all experiments are compared in which antioxidants were applied to blanched diced carrots which were then dehydrated, ground, and stored. Initial pigment content, the

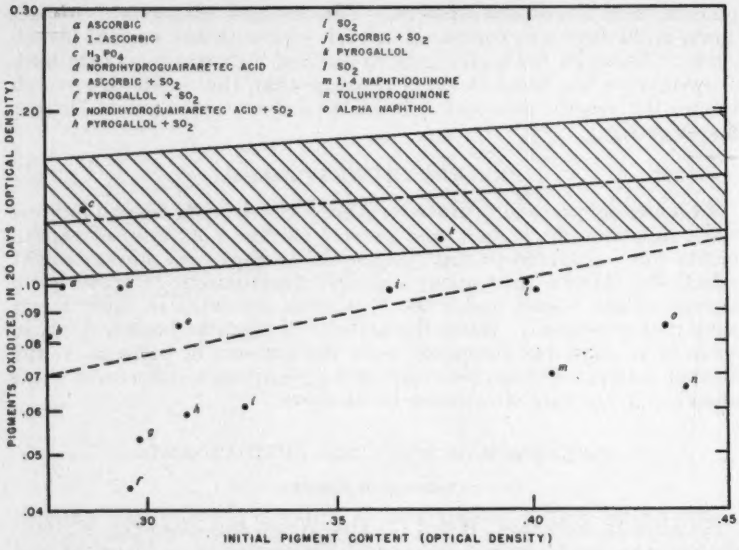

FIGURE 3.—Results of selected antioxidant experiments plotted on the blanch-only treatment curve of figure 2. Points above lined area indicate pigment degradation at a rate faster than blanch only. Points below lined area indicate a retardation in rate of breakdown.

length of induction period, and the pigment oxidized in 20 days are considered. In figure 3 the results of selected runs are plotted on the standard blanch curve shown in figure 2. Attention is called to the following points. The results of the SO_2 treatments fall below the best blanch line, as is to be expected, but in two out of the three cases only slightly so. It seems likely that a statistical analysis of a large number of sulfite treatments would probably indicate a variation of results similar to that observed for blanch only. Such antioxidants as nordihydroguaiaretic acid, ascorbic acid, and pyrogallol when used alone did not afford increased protection of the pigments. When, however, $Na_2S_2O_5$ was added to the pyrogallol so as to make a solution of 0.12 percent $Na_2S_2O_5$ and 0.3 percent pyrogallol in which the blanched carrots were soaked for 5 minutes, the degradation of the carotene pigments was greatly retarded. Toluhydroquinone and 1,4 naphthoquinone, without the addition of $Na_2S_2O_5$ were effective in reducing the rate of pigment break-down.

The pyrogallol used in these experiments was a sample of Merck photographic pyrogallol that had been in the laboratory for at least 15 years. It was slightly tan, indicating a small amount of oxidation. Chemically pure pyrogallol was substituted for the old sample in the late spring of 1947 and, surprisingly, afforded little or no protection

TABLE 2.—*Results of antioxidant treatment of ground, dehydrated carrots*

Treatment after blanch	Initial pigment content	Pigment oxidized in 20 days	Induction period	Days to 80 percent	Relation to blanch
	Optical density	*Optical density*	*Days*		
Soaked in distilled water for 5 minutes	0.42	0.251	3	9	Poor.
	.528	.285	4	10	Do.
	.290	.147	0	8	Do.
	.397	.139	2	12	Do.
Citric acid	.301	.141	0	9	Do.
Tartaric acid	.287	.140	1	9	Do.
Phosphoric acid	.284	.127	0	9	Same.
Ascorbic acid	.280	.101	4	13	Do.
l-ascorbic acid	.275	.082	4	15	Better.
l-ascorbic acid + $Na_2S_2O_5$:					
Before blanch	.305	.094	4	14	Do.
Post blanch	.345	.120	2	12	Same.
Partially dry	.365	.109	5	15	Better.
Before blanch + vacuum	.364	.098	5	16	Do.
Ascorbic acid	.312	.087	0	9	Do.
Ascorbic acid + $Na_2S_2O_5$	.318	.085	0	15	Do.
Pyrogallic acid	.374	.113	0	7	Same.
Pyro + $Na_2S_2O_5$	.311	.059	3	21	Better.
Pyro + $Na_2S_2O_5$:					
Preblanch	.270	.059	5	17	Do.
Post blanch	.296	.044	10	23	Do.
Partial dry	.291	.037	9	25	Do.
$Na_2S_2O_5$	.401	.098			Do.
	.326	.062			Do.
	.396	.103			Do.
N. D. G. A.[1]	.291	.100	0	10	Same.
N. D. G. A. + $Na_2S_2O_5$ [1]	.298	.053	8	22	Better.
Hydroquinone	.328	.117	0	7	Same.
Hydroquinone + $Na_2S_2O_5$	.316	.052	5	21	Better.
Toluhydroquinone	.443	.099	7	19	Do.
Toluhydroquinoue	.440	.066	12	18	Do.
Hydroquinone triacetate	.443	.099	8	19	Do.
1, 2 Naphthoquinone	.423	.095	14	24	Do.
2-4 Methyl-1-4 naphthoquinone	.389	.096	6	14	Do.
b Naphthol	.435	.083	10	20	Do.
1,4 Naphthoquinone	.412	.069	11	21	Do.
1,5 Dihydroxy-naphthalene	.404	.092	5	14	Do.
40 percent Acetone leach	.418	.201	5	11	Poor.

[1] Nordihydroguaiaretic acid.

to the carrot roots being tested. These roots were large and contained an unusually high concentration of carotene pigments. The series of experiments on these high carotene roots, in which blanch, leach, old and new c. p. pyrogallol are compared, is shown in figure 4.

The protection afforded by blanching-alone was somewhat poorer than the average as is shown by the plotting of these points. It is possible that in roots with this large absolute concentration of pigments the rate of break-down after blanching may be higher than the curve of figure 2 would indicate. This series of experiments again indicated that the old sample Merck photographic pyrogallol considerably retarded the rate of pigment degradation. In all, 10 separate experiments, extending over a 2-year period, were run, using this old photographic pyrogallol. In every case considerable protection was afforded the carotene pigments. The authors were unable to discover the reason for the difference in effectiveness of the old and the new c. p. samples of pyrogallol. The possibility was considered that impurities, such as the tannins, might be the factor supplying the protection. Tannic acid (Baker) was tested with negative results.

Under the conditions of our experiments, selected antioxidants, both alone and in conjunction with $Na_2S_2O_5$ afford greater protection than blanch-only and blanch-sulphite treatments to the carotene pigments in dehydrated, ground carrots stored at 40° C.

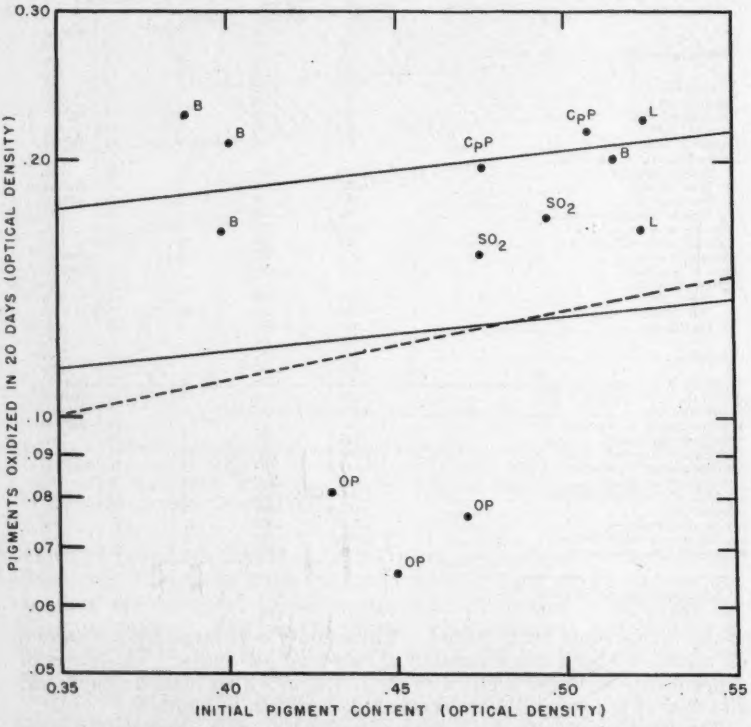

FIGURE 4.—A comparison of blanch-only treatment, (B); $Na_2S_2O_5$, (SO_2), leach (L); c. p. pyrogallol, (CpP), and old Merck photographic pyrogallol, (OP). Points plotted on blanch-only curve. Lower points indicate retardation of pigment break-down.

A study of the degradation of individual pigment components was undertaken to determine whether one antioxidant is more effective than another in protecting any of the pigments. Figure 5 shows the effect of treatment with 0.3 percent $Na_2S_2O_5$ on the break-down of the separate fractions. Here again the alpha and beta carotenes show the same shape curves with the increased induction period characteristic of the sulfite-treated carrots. The treatment apparently affected break-down of the individual components to about the same degree. Similar results were obtained with other antioxidants such as pyrogallol and ascorbic acid.

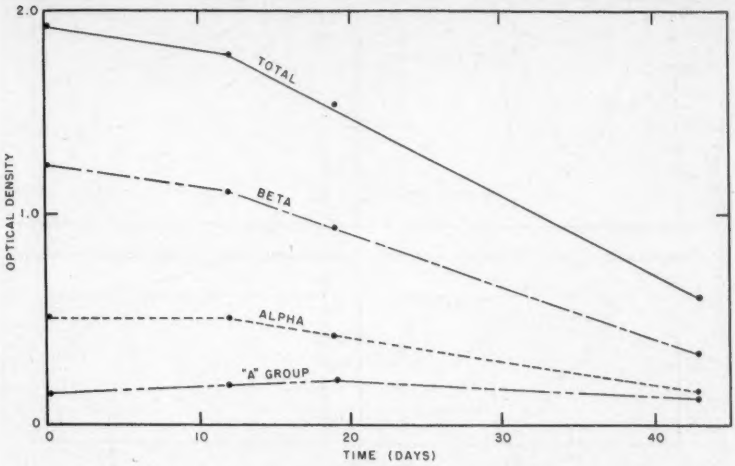

FIGURE 5.—Rate of break-down of total complex and individual pigments after treatment with 0.12 percent $Na_2S_2O_5$. Ground dehydrated carrots stored at 40° C. optical density based on pigment from 1 gram of dehydrated carrots in 200 ml. of solution. Group "A" refers to all carotenoid pigments more strongly adsorbed than beta carotene.

DICED SAMPLES

In testing the effectiveness of antioxidants in protecting the carotenes in dehydrated diced carrots, the only change in procedure from that using ground samples was to store the material as dice for a 3-month period. In conjunction with all experiments on dice, separate 5-gram samples were ground and stored, as in other accelerated tests. Thus, material from a single given processing was stored both in diced and in a ground state. Treatments given the diced carrots are as follows: $Na_2S_2O_5$; $Na_2S_2O_5$+gelatin; $Na_2S_2O_5$+c. p. pyrogallol; $Na_2S_2O_5$+old Merck pyrogallol; c. p. pyrogallol; old Merck pyrogallol; 2-4 naphthoquinone; toluhydroquinone; Na_2SO_3+c. p. pyrogallol; $Na_2S_2O_5$+nordihydroguaiaretic acid; $Na_2S_2O_5$+gallic acid; gallic acid.

In no treatment was the rate of pigment break-down in the dice significantly different from that in the control blanch-only. This was true even after soaking in the 0.03 percent old Merck pyrogallol: 0.2 percent $Na_2S_2O_5$ solution. In the ground aliquot from this same sample of dice the rate of pigment break-down was retarded as usual. The old pyrogallol then is able to protect the pigment in the ground dehydrated carrots but not in the diced carrots.

Figure 6 shows the rates of pigment degradation for blanch-only treatment, $Na_2S_2O_5$, old pyrogallol, and old pyrogallol plus $Na_2S_2O_5$. The upper curve with the low slope represents the rate of pigment break-down in the dice. The differences between these four upper curves are not significant. The left-hand curves in each small graph represent the pigment break-down in the ground material that was

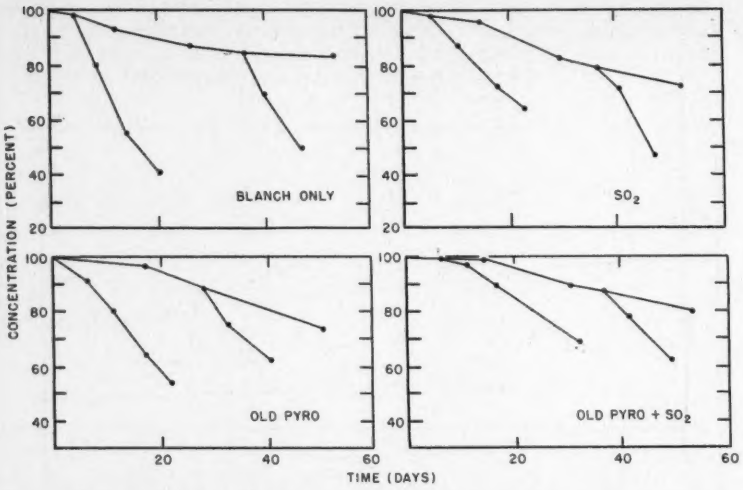

FIGURE 6.—Comparison of rate of pigment break-down in four samples of carrot dice treated as indicated. Upper curves dice stored at 40° C. for 3 months. Left-hand curves represent ground samples stored immediately after preparation. Right-hand curves represent ground samples prepared from dice after 30–40 days' storage.

stored as soon as prepared. Note that the old pyrogallol plus $Na_2S_2O_5$ gives considerable protection. The right-hand curve of each small graph represents the rate of break-down in a sample taken from the diced material after a month's storage. This sample was ground and stored as in all other accelerated tests. Note that in each case these curves are approximately parallel with the curves showing pigment break-down in the ground samples stored immediately after preparation. It seems evident that the rate of break-down and the effectiveness of the antioxidant are greatly influenced by the size of the pieces of stored dehydrated carrots (1).

EFFECT OF PARTICLE SIZE ON PIGMENT STABILITY

While most of the experiments were carried out on ground material and some were carried out on dice, one set of experiments was run on a graded series of particle sizes to determine the effect of particle size on pigment stability. Carrots for this series were prepared, blanched, and divided into two groups. One group was dehydrated without further treatment and the second group was treated with 0.3 percent Merck's pyrogallol prior to dehydration. After drying, the carrots were ground in a coarse food grinder and placed on a series of standard sieves on a Rotap machine. The two sets were thus separated into four particle sizes 28 to 48 mesh, 14 to 28 mesh, 8 to 14 mesh, and greater than eight mesh. The rate of pigment break-down for these eight samples was determined and is shown in figure 7.

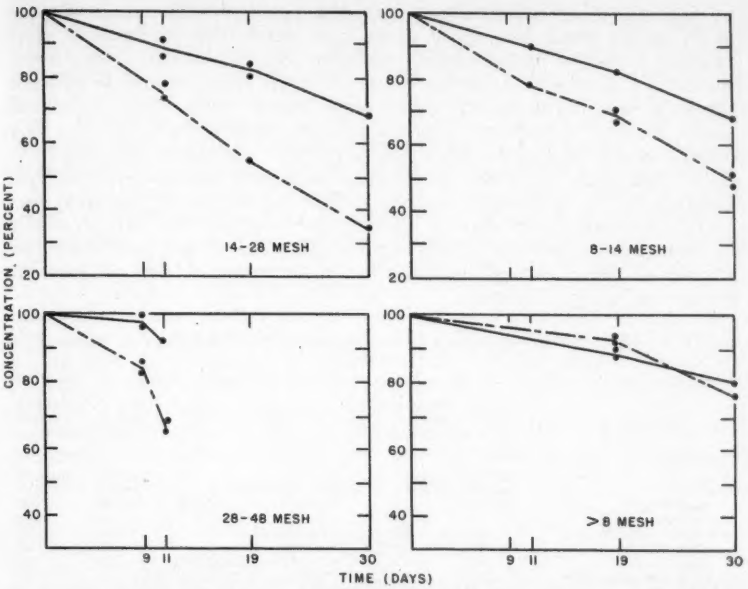

FIGURE 7.—Influence of size of dehydrated carrot particle on rate of pigment break-down. Solid line, in all cases, represents old pyrogallol treatment and broken line, blanch-only treatment.

It is apparent from these curves that the rate of carotene degradation is a function of particle size and that smaller particle have a very rapid break-down. The protective action of pyrogallol (solid line) on this break-down is apparent when the particles are less than 8-mesh in size. However, if the particle size is increased sufficiently there is a decrease in the rate of pigment loss from the untreated pieces but little if any change in the slower rate of break-down of the treated pirces. Apparently the protective action of pyrogallol can be duplicated by increasing particle size but with change in size there is little or no change in effective protection when an antioxidant such as pyrogallol is used.[3]

PREHARVEST SPRAY TREATMENT

A series of preharvest spray treatments of young carrots was undertaken to test the possibility of stabilizing the carotene in the harvested dehydrated material either by changing the phospholipid content or

[3] When the samples for these experiments on diced carrots were prepared, aliquots for $Na_2S_2O_5$ and $Na_2S_2O_5$+old pyrogallol were stored in a basement with a temperature range of 16° to 20° C. These samples have been in storage about 30 months. No analysis have as yet been conducted, but the color of the $Na_2S_2O_5$—only sample is considerably paler than that of the $Na_2S_2O_5$+old pyrogallol. Complete results of this storage test will be reported at a later date.

by incorporating antioxidants into the carrots. Phospholipids are more stable than free fatty acids and while the probability that changes in carrot phospholipids could be affected by spray treatment was low, it seemed that positive results, if obtained, would be of such significance as to make the experiment worth while. The materials used, time of treatment, and results are shown in table 3. The results when compared with the standard control curve figure 1 show that none of these treatments increased the stability of the carotene in the processed carrots sufficiently to be of practical importance although the June 8 choline chloride and the July 27–Aug. 10 kerosene treatments caused significantly less breakdown than the untreated blanches and indicate the desirability of future work in preharvest spray treatment.

TABLE 3.—*Effect of preharvest spray treatments on the breakdown of pigment in carrots*

Spray	Initial pigment content	Pigment oxidized in 20 days	Spray	Initial pigment content	Pigment oxidized in 20 days
	Optical density	*Optical density*		*Optical density*	*Optical density*
Series 1:[1]			Series 2—Continued		
$Na_2H\ PO_4$	0.363	0.145	Glycerine	0.243	0.092
Glycerine	.344	.148	Choline chloride	.282	.121
Choline chloride	.314	.139	Ethanolamine	.231	.089
Ethanolamine	.333	.157	$Na_2S_2O_5$	.246	.103
$Na_2S_2O_5$	.311	.128	Na—glycerophosphate	.215	.095
Na—glycerophosphate	.287	.116	Dreft	.278	.117
Dreft	.316	.117	Series 3:[2]		
No treatment	.315	.118	Kerosene	.183	.095
Kerosene	.214	.070	Kerosene+N. D. G. A	.174	.082
Kerosene+tocopherols	.330	.135	Kerosene+tocopherols	.222	.080
Stove oil	.250	.095	Stove oil	.222	.087
Stove oil+tocopherols	.234	.089	Stove oil+N. D. G. A	.233	.100
Series 2:[3]			Dreft	.262	.113
H_3PO_4	.239	.091	Ethanol amine	.187	.095
$Na_2H\ PO_4$	.252	.098	No treatment	.184	.083
NaH_2PO_4	.265	.101	Choline chloride	.165	.061

[1] Sprayed July 27 and Aug. 10; 84, and 98 days after sowing.
[2] Sprayed July 27; 84 days after sowing.
Sprayed June 8; 35 days after sowing.

SUMMARY

Carotene content varied in different samples of carrots over a 2-year period by 200 percent or more. The initial pigment content does play a parte in rate of carotene degradation. For this reason the ratio of the logarithm of pigment oxidized over a stated period of time to initial pigment content provides the best comparison of rates of break-down between experiments widely separated in point of time.

The pigment in blanched, dehydrated, and ground carrots stored at 40° C. is protected by the following antioxidants to a much greater extent than by blanching only or by blanching plus $Na_2S_2O_5$: pyrogallol (old Merck photographic) plus $Na_2S_2O_5$; nordihydroguaiaretic acid+$Na_2S_2O_5$; toluhydroquinone; and 1,4 naphthoquinone.

No selective action of any antioxidant on any individual carotene component was apparent. In all cases the rate of break-down of alpha and beta carotenes follows that of total pigment complex (crude acetone extract), while concentration of the more strongly adsorbed pigments remains fairly constant.

The rate of pigment break-down in dice is not retarded by the impregnation with antioxidants. Size of dice is the determining factor in rate of pigment break-down and of the effectiveness of certain antioxidants.

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DEVELOPMENT OF PIGMENT IN THE FUR FIBERS OF AGOUTI-COLORED RABBITS[1]

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INTRODUCTION

Fur is made up of two types of fibers: Guard hairs, of which there may be more than one kind, and underfur (*19*).[3] The guard hairs have a greater diameter than the underfur and project beyond the dense covering of the underfur fibers. Ideal pelts possess clear color and have "life." They are silky and full-furred, have sufficient guard-hair coverage, and the skins are of good size. Color plays a very large part in market values since it contributes the key quality to the beauty of furs.

Varying intensities of melanin, the principal pigment found in all animal hair, impart to the fibers colors ranging from black through many shades of gray, and from brown and tans to yellows. Sometimes animal hairs appear to be blue, but on closer examination this color proves to be a form of slate gray. "Tyndall blue" is the term used by Fox (*13*) to describe the blue shade derived from melanin.

Skins that show a pleasing contrast in color between underfur and guard hair are preferred to skins that do not show contrast. In mink farming, one of the objectives is a rich dark-brown over-all color; the clear dark-brown guard hairs contrast with the underfur, which appears to be blue. In various light phases of genetic mutations in mink and fox, the underfur is pale gray or sky blue; the guard hairs are slightly darker. In the silver fox, the black tip of the guard hairs together with the pure white band, located just below this tip, produces a veiling effect; this combination enhances the color contrast. The underfur is gray or blue gray.

Although scattered and fragmentary material is available on pigment studies in the mouse (*12, 22, 30, 31*), guinea pig (*7, 32, 35*), cow (*7*), and sheep (*8*), practically no work of this nature has been reported on animals whose skins are used commercially as furs. In

[1] Received for publication July 7, 1948. This paper is based on a thesis submitted in partial fulfillment of the requirements for the degree of master of arts at the George Washington University.

[2] The author is indebted to Dr. Ira Bowers Hansen of the Zoology Department of the George Washington University for his guidance in the direction of this problem and to Dr. Thora M. Plitt Hardy of the fur fiber laboratory, Bureau of Animal Industry, for her many suggestions.

[3] Numbers in parentheses refer to Literature Cited, p. 532.

August 1944, research on pigment development of the agouti-colored rabbit (*Oryctolagus cuniculus*) [4] was initiated in the Fish and Wildlife Service, United States Department of the Interior, to be carried out in its fur fiber laboratory at Beltsville, Md.[5] In this animal, as in a number of others, the laying down of pigment in alternate dark and light bands produces a mottled pattern. The guard hairs of this rabbit are black at the tip, followed by bands of orange, dark brown or black, and light gray. The underfur is gray for the greater part of its length and is banded at the distal end in most of the fibers.

The agouti-colored rabbit was selected as the animal for study in this investigation for several reasons. Since the guard hair is tipped with black, there is a heavy concentration of melanin in the early stages, which facilitates the study of pigment development in the fur fiber. Also, the gestation period in the rabbit is short, approximately 31 days, and embryos can be obtained within relatively short periods of time. This animal is readily available, is docile in temperament, and can be handled without much difficulty.

MATERIAL AND METHODS

To study the sequence of pigment development in the agouti-colored rabbit, blocks of tissue were removed from two fetuses taken at 18 and 25 days; from nine juvenile rabbits at 1 to 5 days and at 8, 10, 12, and 25 days after birth; and from a live adult animal;[6] age unknown.

Bouin's, 5-percent formalin, and Zenker's (*9*) were used as fixatives. Bouin's, according to the method outlined by Becker and Roudabush (*1*), gave best results. Tissue blocks were cut at an angle to the skin surface and oriented to obtain longitudinal sections of the hair follicles. It was not always possible to get exact orientation, with the result that hairs sometimes appeared in cross or tangential section. If freshly cut paraffin sections are placed on a slide and the paraffin removed by applying a few drops of xylol, the presence of pigment in the hairs can be used as a guide in determining the proper orientation.

Various stains were used in this investigation. Haematoxylin and eosin show up the structures of the skin, but haematoxylin masks the pigment to some extent. Cell outlines are also well defined with iron haematoxylin and orange G. Silver nitrate was used when it was desired to intensify the appearance of pigment granules, and the slides were sometimes counterstained with pyronin methyl green (*23*). The orcein and Giemsa stain described by Pinkus (*29*) proved to be valuable in the definition of the inner root sheath. Mallory's triple connective tissue stain (*17*) and methyl green were also used.

As a mounting medium, gum dammar was superior to Canada balsam since it dried faster and seemed not to yellow with age.

The dopa (shortened term for dihydroxyphenylalanine) reaction as worked out by Bloch (*5*) was used to determine the potentialities of

[4] The agouti-colored rabbits were provided through the courtesy of Dr. Don R. Coburn, Patuxent Research Refuge, Bowie, Md.

[5] All the activities pertaining to the production of fur animals in captivity (fur farming), including domestic rabbits, were transferred to the U. S. Department of Agriculture June 30, 1946, by act of Congress.

[6] Blocks of skin from the adult rabbit were excised by Dr. Louis C. Heemstra of the Bureau of Animal Industry.

pigment development, requiring the use of fresh tissue or tissue fixed for a short time in 5-percent formalin. Blocks of skin 3 to 5 mm. thick were fixed in formalin from 2 to 3 hours. Sections ranging in thickness from 15 to 30μ were then cut on the freezing microtome (*14*), rinsed in distilled water, and placed in the dopa solution (*25*). Approximately 20 cc. of the solution was used for 50 sections and kept at 40° C. for 3 hours, with a fresh change of dopa solution after the first half hour and then again after another hour (*32*).

The paraffin section method for the dopa reaction, developed by Becker, Praver, and Thatcher (*3*), revealed more nonspecific blackening around the outer edges of the tissue. Blocks of skin were first treated with the dopa solution at 37° C. for 15 hours, with a change of solution after the first half hour. Following this treatment, the blocks were fixed in Bouin's, carried through the alcohols and xylol, and embedded in paraffin.

PIGMENTATION IN THE HAIR

18-DAY-OLD FETUS

No pigment is evident in the 18-day-old fetus. At this stage, the development of hair is more advanced in the ear (fig. 1) than in other regions of the body. Knoblike aggregations of cells, the hair anlagen, are present in the epidermis, and these cells as they grow downward into the dermis become enclosed in a pocket, the hair follicle. In some cases it appears as though the proliferation of cells of the hair proper gets under way before the definitive papilla takes shape (*28*).

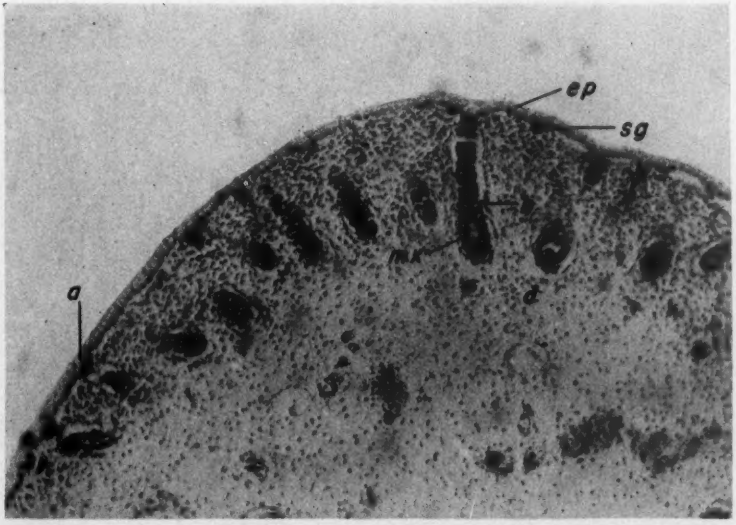

FIGURE 1.—Anlagen and early hairs in ear of 18-day-old fetus. The matrix has taken up the stain (haematoxylin and eosin) more deeply than the surrounding cells: *a*, Hair anlage; *ep*, epidermis; *sg*, columnar cells of stratum germinativum; *h*, downgrowth of hair into dermis; *mx*, matrix; *d*, dermis. × 100.

The papilla is produced by mesenchymal cells and fits into the broadened base of the hair. The mesenchymal cells surrounding the hair root become the connective tissue sheath; this sheath is found only in the guard hairs and usually serves for the insertion of smooth muscle fibers.

The matrix cells of the hair are located in the bulb, just above the papilla. These are the active cells that later give rise to the hair, the inner root sheath, and the pigment cells themselves. The hair and its sheaths—the inner root sheath and the outer root sheath (fig. 3)—are epidermal in character, whereas the connective tissue sheath (fig. 4) and the papilla (figs. 6 and 8) are dermal in origin.

25-DAY-OLD FETUS

Generally, the first pigment cells are seen in the matrix region of the hair in the 25-day-old fetus, but occasionally some may be found in the undifferentiated medulla. These cells are also called melanoblasts and differ from the other epithelial cells in the hair only in that they may carry pigment. Melanin granules in some cases are found along the periphery of the cell (fig. 2), but usually they fill the entire cell (fig. 3). In the study of wool from colored and from white sheep, Hardy and Plitt (*18*) found that the smallest visible units of structure were granules or "particles." These particles were colorless in the white, Lincoln breed of sheep, whereas in the colored, Karakul sheep, they were both colorless and pigmented, the latter being identical with the familiar pigment granules.

At the 25-day fetal stage, the columnar cell make-up of the outer root sheath is clearly recognizable. In figure 4 the outer root sheath

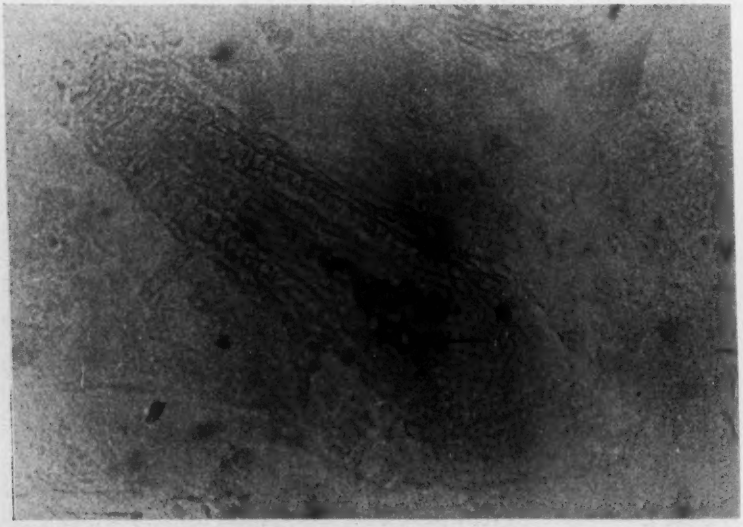

FIGURE 2.—Skin section from 25-day-old fetus; taken from the head and stained with orange G. The pigment granules are arranged along the periphery of the melanoblast, indicated by the arrow. × 400.

FIGURE 3.—Skin section from 25-day-old fetus; taken from the head and stained with orange G: *or*, Outer root sheath; *ir*, inner root sheath; *mel*, fully pigmented melanoblast; *d*, dermis. × 400.

FIGURE 4.—Early development of hair in skin from head of 25-day-old fetus; stained with haematoxylin and eosin: *sg*, Basal cell layer of epidermis (stratum germinativum); *irt*, tip of inner root sheath; *or*, outer root sheath; *mm*, melanoblast in matrix; *cts*, connective tissue sheath; *d*, dermis. × 200.

can be traced to the basal layer of the stratum germinativum of the epidermis and is seen to be continuous with it.

JUVENILES (1 TO 25 DAYS OF AGE)

In newborn rabbits of the pigmented breeds the color contained in the hairs that lie just below the skin surface is visible through the skin. Rabbits are born naked; however, a sparse covering of hairs that have pierced the skin can be observed with a hand lens. In figure 5 hairs can be seen emerging from some of the follicles of a 1-day-old rabbit. The young are covered with a good hair growth at about 5 days of age.

In the agouti-colored rabbit, histological sections from the juvenile, as well as from the adult, show that the melanoblasts are always rounded in appearance (fig. 6). Branched pigment cells, however, have been reported in the gray rabbit (*26*) and in other animals (*6*, *20*, *27*, *34*).

The cells in the central portion of the matrix pile up in rouleaux to form the medulla of the hair (figs. 7 and 8). In surface view these cells are round, whereas in end view they appear flat and produce a banded effect. In the rabbit the pigment in the medullary cells is granular in structure; both granular and diffuse pigments are found in the cortex (fig. 9). Although granular and diffuse pigments are present in the hairs of most fur-bearing animals, only one form of pigment has been reported for members of some species (*15*, *31*), and this may be either diffuse or granular.

FIGURE 5.—Hair follicles in skin section from loin of a 1-day-old rabbit; stained with haematoxylin and eosin. Arrow points to hair emerging from follicle. × 100.

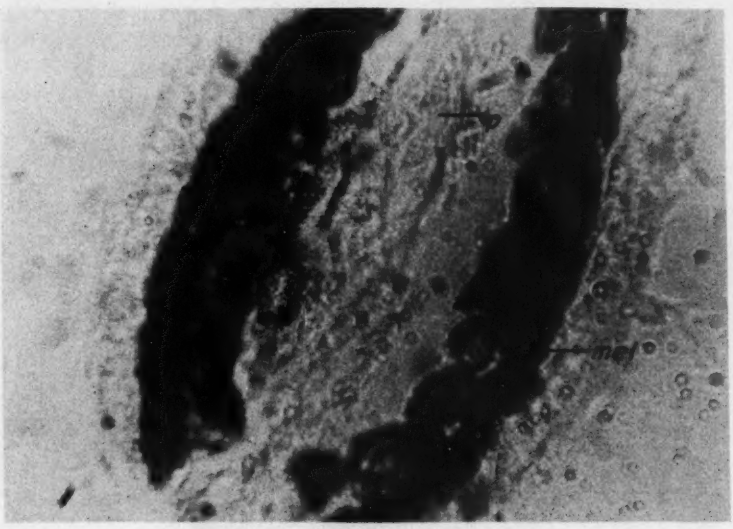

FIGURE 6.—Root end of a hair in the unstained skin from loin of a 2-day-old rabbit. Pigmented cells in the bulb are rounded: *p*, Papilla; *mel*, melanoblast. × 900.

The aggregation of melanin granules (fig. 10), often spoken of as a cap, that appears above the nucleus in the medullae in many animal hairs has led some authors to suggest that melanin is nuclear in origin (*8*). Just what role the nucleus or the cytoplasm plays in melanin elaboration is still to be determined. Conklin (*10*) believes that all cellular differentiations may be formed by the reconstruction of substances that pass through the nuclear membrane and that these substances then enter the cytoplasm in solution.

Boyd (*8*) speaks of single and double nuclear caps of pigment granules in sheep. In some hairs of the agouti-colored rabbit the cap of melanin granules is found at the top and apparently at the bottom of the nucleus (fig. 10).

In skin sections of some of the early stages, pigmented cells were observed to align themselves in a way that suggested polarity. It may be that this cellular polarity (*10*) influences the orientation of pigment granules and that this polarization is responsible, in part, for the so-called capped nucleus. In figure 11 the pigment granules are concentrated at the distal end of the pigment cells. As the hair approaches the skin level, there is a shrinkage of cytoplasm, and air spaces make their appearance between the medullary cells (*34*).

The laying down of dark-colored granules followed by the deposition of light-colored granules was observed in some of the fibers. The difference in intensity (*8, 11, 13, 35*) is illustrated in figure 12; microscopically the light-colored melanin granules appear yellow or light brown.

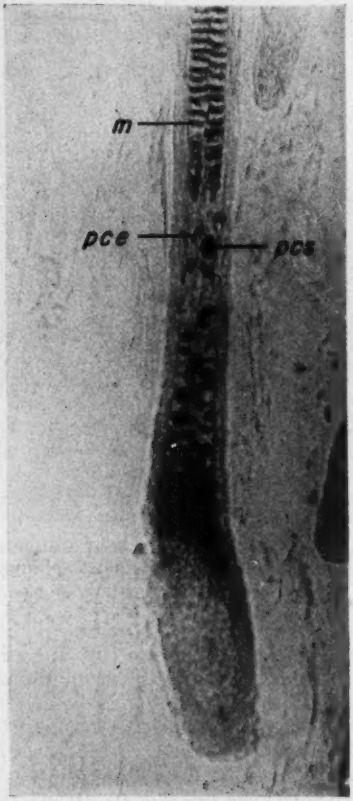

FIGURE 7.—Pigment cells in the form of rouleaux in the medulla of hair of a 2-day-old rabbit. Skin section was taken from the loin and stained with methyl green: *m*, Medulla; *pce*, pigment cell in end view; *pcs*, pigment cell in surface view. × 100.

ADULT

The replacement of hairs in the adult rabbit is different from the development of fetal hairs in that new anlagen no longer appear in the basal layer of the epidermis and grow downward into the dermis. Hairs are now differentiated from epithelial cells already established in the dermis. That new hairs arise from the already existing follicles has been reported by several authors (*33*, *34*). It is believed that the new hair germ is formed from material of the outer root sheath. This sheath in the fetal stages is seen to be continuous with the stratum germinativum. The part the papilla plays in hair regeneration has been disputed. Hertwig (*21*) is of the opinion that a new papilla is formed, whereas Trotter (*34*) believes that the new hair is nourished by the enlarging old papilla or by a newly formed papilla.

FIGURE 8.—Guard hair in skin from dorsum of a 10-day-old rabbit. Cells proliferated from the matrix form the medullary columns. Mallory's triple connective tissue stain was used: *c*, Cortex; *m*, medulla; *p*, papilla, *mx*, matrix; *f*, fat cells. × 200.

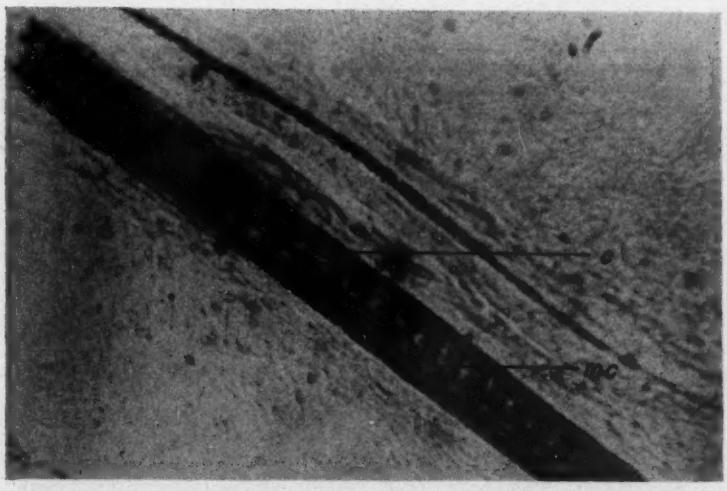

FIGURE 9.—Diffuse and granular pigment in cortex of hair from 2-day-old rabbit. Skin section was taken from the region of the loin and stained with methyl green: *c*, Cortex; *mc*, outline of medullary cells seen through the cortex. × 200.

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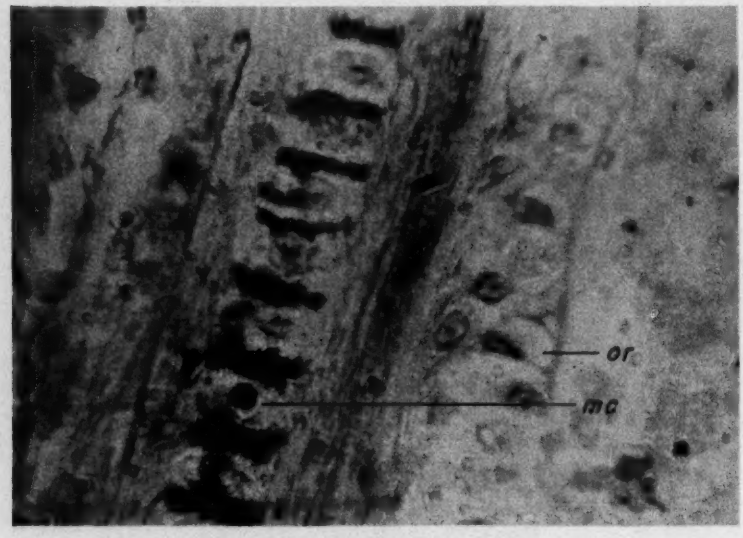

FIGURE 10.—Hair in the skin of 10-day-old rabbit, taken from the dorsum, showing nuclei in the medullary cells. These cells later keratinize and the nuclei disappear. Stained with haematoxylin and eosin: *or*, Outer root sheath; *mc*, medullary cell with caps of melanin granules above and apparently below the nucleus. × 900.

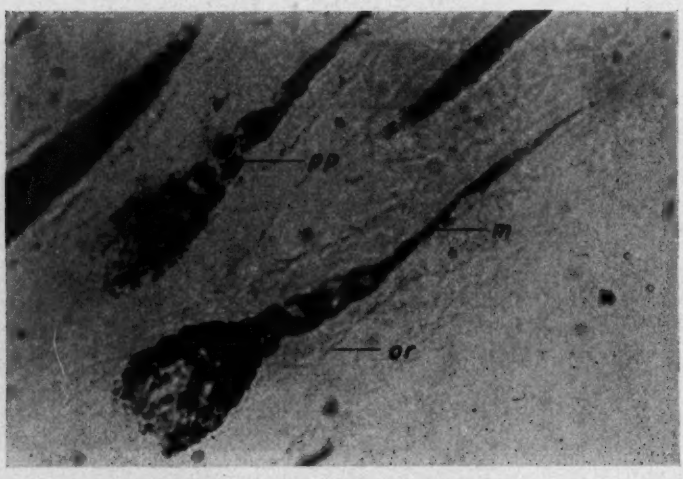

FIGURE 11.—Longitudinal section of unstained skin from 2-day-old rabbit ear showing polarity of pigment granules: *pp*, Pigment cells exhibiting polarity; *m*, medulla; *or*, outer root sheath. × 400.

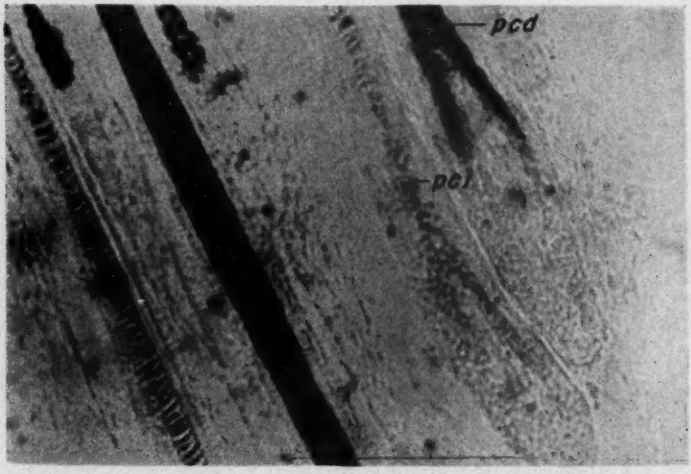

FIGURE 12.—Light- and dark-colored melanin in 2-day-old rabbit hair (unstained), taken from the dorsum: *pcd*, Dark-colored pigment cells; *pcl*, light-colored pigment cells. × 200.

In the young juvenile the hair covering is fairly uniform, apparently consisting of only one type of fiber. These fibers are softer in texture than those found in the adult. In the coat of the older juvenile and in the adult there is a distinction in the hair types (*19*). The guard hairs are the longer hairs, thicker than the underfur (fig. 13, *A*), and contain two or more columns of medullary cells (fig. 13, *B*). The underfur fibers are more numerous and contain but one medullary column.

When the hair above the skin measures approximately 3 cm. in length, the papilla diminishes in size, and the hair enters a resting phase. If the skin of a rabbit whose pelt is prime is examined by blowing into the fur, or by parting the fur, the skin is observed to be creamy white and devoid of pigment spots since the root ends of the hairs have completed their growth cycle and are free of pigment. This period of inactivity is followed by one of renewed differentiation in the hair follicles, and pigment spots (fig. 14) can be seen through the skin (*16*), an indication that pigment is being deposited in the new incoming hairs. The new hairs, after a period of growth, pierce the skin and for a time lie side by side with the club, or mature, hairs. The new short hairs can be observed macroscopically by running the hand along the body of the animal in a direction opposite to the natural hair flow.

The club hairs that are being shed are recognized by the frayed appearance at the root end (fig. 13, *A*). Pinkus (*28*) states that the hair forms a brushlike or clublike structure because it is no longer compressed by Henle's layer at its lower end. Huxley's and Henle's layers, subdivisions of the inner root sheath, usually remain attached to the shedding hair.

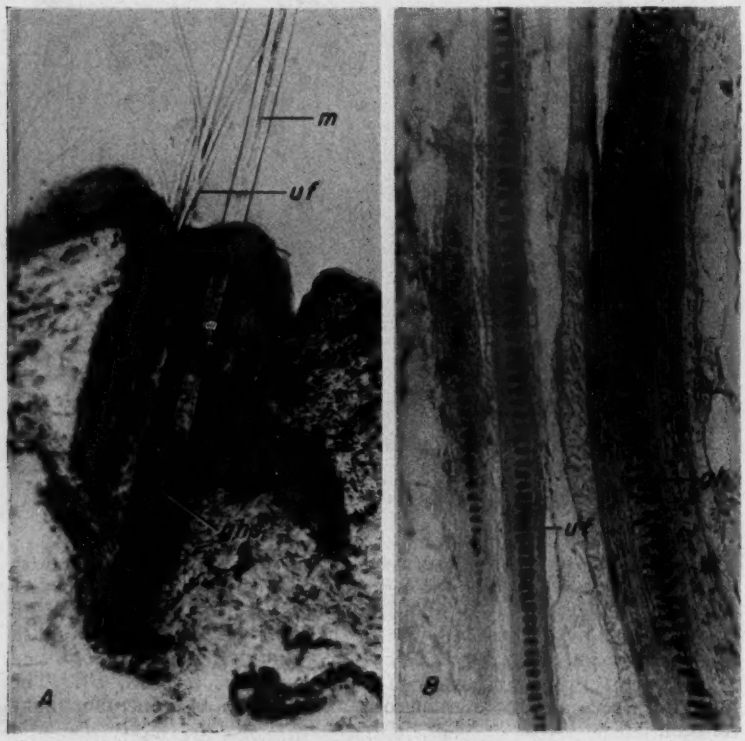

FIGURE 13.—*A*, Guard hair and underfur fibers from the loin region of an adult rabbit. The guard hair shown is in the process of being shed; attention is called to the brushlike structure at the root end. Stained with haematoxylin and eosin. × 100. *B*, Guard hair and underfur fibers from dorsum of 10-day-old rabbit. Stained with Mallory's triple connective tissue stain. × 200. *m*, Base of the medulla in guard hair; *ghs*, brushlike structure of shedding hair; *gh*, guard hair; *uf*, underfur.

PIGMENT IN THE SKIN

In untreated slides, i. e., slides which are not tested for the dopa reaction, pigment in the skin was observed only in the basal layer of the epidermis in the rabbit ear. The two layers of the epidermis in figure 15 can be easily distinguished. The outermost layer, the stratum corneum, is the most highly keratinized and the cells nearest the surface are constantly being sloughed off. The basal cells of the stratum germinativum, the innermost layer, supply the rest of the epidermis with new cells. Pigment was found nowhere else in the epidermis and, except for the sections treated for the dopa reaction (figs. 16 and 17), was never observed in the dermis.

Both dermal and epidermal pigmented cells have been reported for man, monkeys, some guinea pigs, the gray rabbit, and the gray house

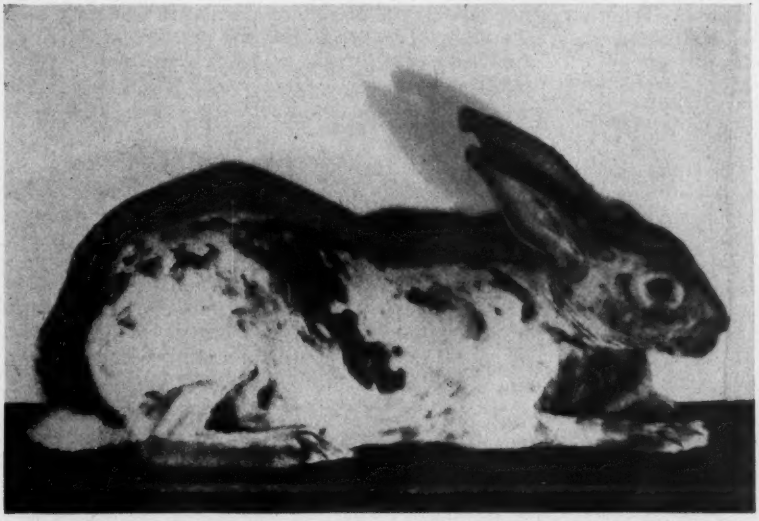

FIGURE 14.—Right side of adult agouti-colored rabbit showing newly developed pigment spots. The hair of this side was removed by means of an electric clipper.

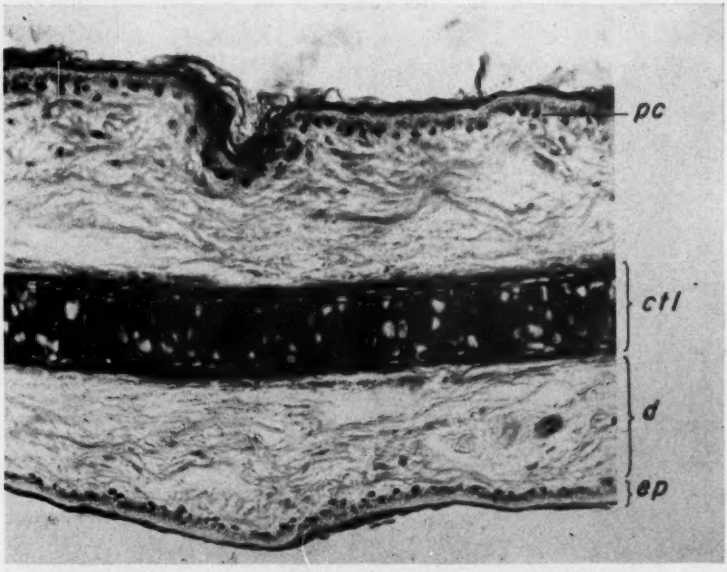

FIGURE 15.—Pigment cells in the skin from the ear of a 25-day-old rabbit; stained with orcein and Giemsa: *pc*, Pigment cells in stratum germinativum; *ctl*, cartilage; *d*, dermis; *ep*, epidermis. × 100.

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mouse. Whether or not these pigmented dermal cells are true melanoblasts is uncertain. Laidlaw (*24*) states that the pigmented cells in the dermis are cells that have taken up, or phagocytized, melanin on their way to the lymphatics.

DOPA, THE TEST FOR MELANIN

Melanin, because it is highly insoluble, is difficult to isolate. Many substances have been proposed as possible substrates, among which are derivatives of pyrocatechin (*6*) and pyrrol. The most widely accepted hypothesis today is that the formation of melanin is an oxidative process in which an enzyme in the pigment cell itself acts on a substance brought to the cell via the circulating blood. Bloch (*5*) and his supporters (*2*, *26*) call the enzyme dopa-oxidase and consider the melanogen, or colorless precursor, to be a substance closely related to dopa. However, the specificity of the enzyme has been questioned (*4*) since the reaction is also given by some tissue elements not connected with pigment formation, such as parts of the sweat gland and leucocyte granules.

The purpose of the dopa reaction is to make evident the production capacity of melanin in the skin or hair. At the time this investigation was undertaken, it was intended to apply the dopa solution to tissue sections of several fetal stages to observe, if possible, the time of pigment determination in the rabbit. However, the material worked with first was of the adult type, and when this work was completed the supply of dopa powder on hand was exhausted. A new supply of the powder was unobtainable. Reed and Sander (*30*) found from grafts

FIGURE 16.—Longitudinal section of 4-day-old rabbit skin from the loin, treated with dopa: *dp*, Dopa-positive hair bulbs; *dpm*, dopa melanin in dermis. × 100.

FIGURE 17.—Longitudinal section of skin from adult rabbit shoulder, cut on the freezing microtome and treated for the dopa reaction: *ep*, Epidermis; *du*, upper level of dermis; *dl*, lower level of dermis. × 100.

that, although pigment granules in the mouse appear on the twenty-first or twenty-second day, the pigmentation pattern is determined by the seventeenth or eighteenth day.

The dopa solution when applied to adult skin tissue, in the manner previously described, causes a good deal of nonspecific blackening, especially around the outer edges of the tissue. The test is positive in the bulbs of growing hairs (fig. 16) and negative in mature or shedding hairs. The amount of melanin produced by the dopa reaction is not necessarily the amount normally activated. The longer the tissue is immersed in the solution, the darker will be the resulting reaction.

Dopa melanin was also observed in the lowermost layer of the epidermis and in the upper and lower levels of the dermis (fig. 17). The significance to be attached to the reaction in the dermis is difficult to interpret since no dermal pigment was observed in any of the slides made of nontreated tissue. In some cases, when blood vessels or capillaries were present, it was noticed that the dermal dopa pigment tended to concentrate near these structures.

SUMMARY

In a study of the sequence of pigment development in the short-haired rabbit of the agouti or barred pattern, skin sections were made of two fetuses 18 and 25 days old, nine juveniles from 1 to 25 days old, and one adult rabbit.

No histological evidence of pigment was found in the 18-day-old fetus; hair primordia, however, were clearly established. The first indications of the pigment cells, the melanoblasts, show up in sections

of the hair of the 25-day-old fetus. These cells are rounded in form and are located in the matrix of the hair bulbs.

Rabbits are covered with a good growth of hair within approximately 5 days after birth. The development of melanin proceeds rapidly and the pigment cells, as they are moved upward from the bulb, take part in the formation of the medulla and the cortex. Only granular pigment was observed in the medulla, whereas both granular and diffuse pigments are present in the cortex. By the time the hairs approach the level of the skin, the nuclei have become smaller owing to a shrinkage of the cytoplasm and air spaces appear between the cells of the medullae. This phenomenon is responsible for the ladder-like appearance of the medullary cells.

The orientation of pigment granules to form a cap above, and perhaps in some cases below, the nucleus in the medullae of rabbit hairs may be influenced to some extent by the cellular polarity of the melanoblasts.

The difference in intensity of melanin at the time the granules are laid down accounts for the differences in color of the fibers.

In the unprime pelt, pigment spots are indicative of the deposition of melanin in new hairs that are being formed just below the skin surface. Mature hairs contain no pigment at the root end, and it is for this reason that the skin of a prime pelt is creamy white and free from pigment spots.

When the dopa test, devised by Bloch to check on the potentialities of pigment development, was used, the reaction in the adult rabbit was positive in the bulbs of the growing hairs and negative in mature, or shedding, hairs. Dopa melanin was present in the dermis and in the lowermost layer of the epidermis of the dopa-treated sections. However, in the untreated sections no melanoblasts were found in the skin of the rabbit except in the basal layer of the epidermis of the ear.

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COMPLEMENTARY FACTORS FOR DARK-RED PLANT COLOR IN UPLAND COTTON[1]

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INTRODUCTION

Red plant color in upland cotton (*Gossypium hirsutum* L.) was found by McLendon (*3*)[3] and Ware (*8*) to be due to a single incompletely dominant factor. A series of allelomorphic factors governing intensity and distribution of red color, or anthocyanin pigmentation, in *G. hirsutum* and other New World ($n=26$) cottons was later reported by Harland (*1*), who found that the factor for *hirsutum* red was not, however, a member of this series but was a duplicate gene. In the Asiatic ($n=13$) cottons the gene for red plant color was reported by Silow and Yu (*6*) to be a member of an extensive anthocyanin allele series.

A red plant-color mutation originating as a red chimera on a normal green plant of the Acala variety of upland cotton at Shafter, Calif., was described by McMichael (*4*). He found that dwarfing was apparently completely associated with the red plant color and that the F_2 from a cross of the dwarf red mutant with normal green plants segregated into a 1 : 2 : 1 ratio, demonstrating incomplete dominance for dwarfing and red color. Another dwarf red mutation occurring as a red chimera on a normal green plant of the upland variety Cook 912 was found by D. M. Simpson at Knoxville, Tenn., in 1939. The former mutation is designated as dwarf red Acala and the latter as dwarf red Cook 912.

The present paper describes a dark-red plant color which appeared in a stock of red Acala cotton of normal stature at Stoneville, Miss., and presents the results of studies which show that the inheritance of dark red may be interpreted in terms of the factor for *hirsutum* red and a factor for dwarf red.

[1] Received for publication July 19, 1948. Cooperative investigations by the Division of Cotton and Other Fiber Crops and Diseases and the Delta Branch of the Mississippi Agricultural Experiment Station. Paper No. 153, New Series, Mississippi Agricultural Experiment Station.

[2] The writer is indebted to J. W. Neely, J. B. Dick, A. S. Ramsey, and the late S. G. Brain, who made the crosses and classified some of the F_2 and backcross progenies reported in this study.

[3] Italic numbers in parentheses refer to Literature Cited, p. 543.

MATERIALS AND METHODS

A stock of red Acala cotton of normal stature was transferred from Shafter, Calif., to Stoneville, Miss., and was planted there in 1937. The following year a progeny obtained by selfing a single plant of this stock contained two darker red segregates, which on continued selfing and selection produced a homozygous phenotype referred to here as dark red. The homozygous plants were about half the usual height of green or red Acala. They were of a greatly intensified blackish-red anthocyanin color throughout (including the bolls). The dorsal surface of the leaves was deep red in contrast to the green dorsal surface in red Acala and other red strains of cotton.

Dwarf red Cook 912 was introduced to Stoneville in 1939, and dwarf red Acala in 1942. These stocks were similar to dark red in plant size but were lighter pigmented, being of a color lighter than *hirsutum* red as represented by the red Acala and Winesap stocks. Dwarf red Cook 912 was somewhat smaller and darker red than dwarf red Acala.

In 1944 a single plant of dark red used as the pollen parent was crossed with normal green Deltatype Webber, an upland variety. The same dark-red plant was crossed with the linkage tester $R_1^{RO}L^O$ Lc_1^KFn. (In conformity with the symbols proposed by Hutchinson and Silow (*2*), R_1^{RO} here designates red plant color, L^O okra leaf shape, Lc_1^K brown (khaki) lint color, and *Fn* naked (not fuzzy) seed.) The F_1 progenies from these crosses were selfed and were backcrossed, in the first case to normal green and in the second case to the linkage tester. The F_2 and backcross populations were classified as to plant color before the stands were thinned and again after the thinning. Later the F_2 and backcross progenies from the crosses involving dark red and the linkage tester were classified as to all the characters of the tester.

Near the end of the growing season, plant height measurements were made in the F_2 population from the cross dark red $\times$ normal green Deltatype Webber to evaluate the dwarfness which appeared in some of the color phenotypes.

To check the F_2 classification in both series of crosses, all plants were selfed. For each phenotype the number of progenies grown in the F_3 was as far as possible proportionate to the observed frequency in the thinned F_2 stand, the desired number of progenies for the F_2 class lowest in frequency being 10. The F_3 progenies were classified as to plant color only.

In 1945 dwarf red Acala and dwarf red Cook 912 were crossed with the linkage tester. The F_2 population from each of these two crosses was classified as to plant color. The populations obtained by backcrossing the F_1 of each cross to the tester were classified as to both plant color and leaf shape.

In the same year dwarf red Acala was crossed with dwarf red Cook 912. The F_1 population from this cross was compared with the parental stocks as to plant height and plant color. The F_2 population was classified as to plant color only.

INHERITANCE OF THE DARK-RED CHARACTER

F_2 AND BACKCROSS TESTS

Dark red when crossed with normal green Deltatype Webber produced an F_1 about intermediate in height between the two parental stocks but having a red color about intermediate between that of red Acala and dark red. In the F_2 seven color phenotypes were distinguished. These and their plant frequencies are given in table 1.

The plants of the red phenotype resembled red Acala. The dwarf red plants, the red factor of which is designated by the symbol *Rd*, were somewhat lighter than the red in color and differed from them in pigment distribution, having less color on the leaf blades, especially around the stomata, but having deeper red petioles and leaf veins. The green plants resembled the normal green parent. With respect to color intensity the intermediate-red phenotype fell between the red and the green and the intermediate-dwarf-red phenotype fell between the dwarf-red and the green. The intermediate-dark-red phenotype represented the recovered F_1 type and was distinctly darker than either the dwarf-red or the red. The dark-red phenotype showed somewhat more variation in color and in plant height than the parental type.

TABLE 1.—*Observed plant frequencies, mean plant heights near end of growing season, theoretical genotypes and genotype ratio, and Rd factor classes [1] for 7 F_2 phenotypes from the cross dark red × normal green*

F_2 phenotype	Observed plant frequency	Mean plant height	Theoretical genotype	Genotype ratio	*Rd* factor class
	Number	*Inches*			
Dark red	146	31.53±0.69	$R_1^{RO}R_1^{RO}Rd\ Rd$ ...	1	[2]
			$R_1^{RO}R_1^{RO}Rd\ rd$ ...	2	
			$R_1^{RO}r_1^{RO}Rd\ Rd$ ...	2	
Intermediate dark red	196	40.46± .48	$R_1^{RO}r_1^{RO}Rd\ Rd$ ...	4	1
Red	60	52.80± .98	$R_1^{RO}R_1^{RO}rd\ rd$ ...	1	0
Intermediate red	102	54.32± .77	$R_1^{RO}r_1^{RO}rd\ rd$ ...	2	0
Dwarf red	26	28.12±1.90	$r_1^{RO}r_1^{RO}Rd\ Rd$ ...	1	2
Intermediate dwarf red	85	40.40± .83	$r_1^{RO}r_1^{RO}Rd\ rd$ ...	2	1
Green	69	52.42±1.03	$r_1^{RO}r_1^{RO}rd\ rd$ ...	1	0

[1] *Rd* factor class=average number of *Rd* (dwarf-red) factors.
[2] Theoretical average number of *Rd* factors is 1.6. It was rounded for convenience.

The theoretical F_2 genotype ratio presented in table 1 was calculated on the assumption that 2 pairs of complementary factors for anthocyanin color were involved, 1 pair in the homozygous condition ($R_1^{RO}R_1^{RO}$) giving red, the other pair in the homozygous condition (*Rd Rd*) giving dwarf red, and 1 member of each in the double-heterozygous condition ($R_1^{RO}r_1^{RO}Rd\ rd$) giving the more intense color intermediate dark red, and the further assumption that plants with 3 color factors ($R_1^{RO}R_1^{RO}Rd\ rd$ or $R_1^{RO}r_1^{RO}Rd\ Rd$) were not distinguished from those with 4 color factors ($R_1^{RO}R_1^{RO}Rd\ Rd$). While the observed frequencies approach this ratio, the obtained χ^2 value, 57.96, shows a poor fit (P=less than 0.01). However, from a backcross of the F_1 ($R_1^{RO}r_1^{RO}Rd\ rd$) to the normal green parent ($r_1^{RO}r_1^{RO}rd\ rd$) frequencies of 27 intermediate dark red, 33 intermediate red, 35 intermediate dwarf red, and 29 green were obtained. These frequencies show a good fit (χ^2=1.29, P=0.50–0.95) to the 1 : 1 : 1 : 1 ratio expected from the segregation of 2 independent pairs of factors.

It may readily be determined from table 1 that the dwarf-red and dark-red phenotypes alone fell short of the expected frequencies. This, undoubtedly, was due largely to the failure of some plants with two *Rd* factors to survive to the time of classification. This fact alone could account for the poor fit of the F_2 ratio. The backcross progeny, on the other hand, did not include any phenotypes homozygous for *Rd*. To obviate similar difficulties in the next generation, F_3 progenies were classified as early as practicable.

Measurements taken near the end of the growing season showed the phenotypes to differ in plant height (table 1). The analysis of variance (table 2) brings out that these differences were highly significant ($F=142.73$). From table 1 it appears that the differences in plant height between phenotypes were associated with differences in number of *Rd* factors involved, the phenotypes with no *Rd* factors (class 0) having the greatest plant height while those with two *Rd* factors (class 2) had the least. The analysis of variance (table 2) shows, in fact, that the differences in plant height between phenotypes were due almost entirely to height differences between *Rd* factor classes, which were highly significant ($F=424.58$) while height differences between phenotypes within *Rd* factor classes were not significant ($F=1.80$). This suggests that dwarf stature is closely associated with this factor for red plant color.

TABLE 2.—*Analysis of variance for plant heights of the F_2 population from the cross dark red × normal green*

Source of variation	Degrees of freedom	Variance	*F* value
Between phenotypes	6	8,589.22	142.73**
Between *Rd* factor classes	2	25,551.32	424.58**
Within *Rd* factor classes	4	108.17	1.80
Within phenotypes	677	60.18	
Total	683		

**Significant at the 1-percent point.

The dark-red phenotype, classed as having 2 *Rd* factors (table 1), theoretically averaged only 1.6, because two plants out of five were of the genotype $R_1^{RO}R_1^{RO}Rd\ rd$. The difference of 0.4 factor between 1.6 and 2, the number of *Rd* factors for the dwarf-red phenotype, was associated with a mean difference in plant height of 3.41 inches, which in terms of its standard error is not significant ($t=1.69$).

The F_2 from the cross of dark red with the linkage tester consisted of 205 dark-red and 80 red plants, frequencies showing a good fit ($\chi^2=1.43$, $P=0.20$–0.50) to the 3:1 ratio expected where dark red and the linkage tester are both homozygous for red. These results identified the red factor involved in dark red with that for *hirsutum* red, R_1^{RO}, previously reported by McLendon (*3*), Ware (*8*), and others under different symbols.

F_3 PROGENY TESTS

For 4 of the F_3 progenies from the cross of dark red with normal green, it was observed that the F_2 phenotype classification had been incorrect. Out of 9 progenies from plants classed as dwarf red, 3

segregated as intermediate dwarf red; and out of 36 progenies from plants classed as intermediate dwarf red, 1 proved to be of dwarf red parentage. The failure to distinguish accurately between these phenotypes probably explains the high standard error of the mean height shown for dwarf red in table 1. The results of classifying the F_3 progenies other than the 4 misidentified are presented in table 3.

As expected from the genotypes postulated in table 1, three of the phenotypes listed in table 3—red, dwarf red, and green—showed no segregation in the F_3. The intermediate red and intermediate dwarf red each segregated into three phenotypes, the frequencies of which showed a close fit to the ratio 1:2:1. The intermediate dark red segregated into seven phenotypes, the frequencies of which showed a good fit to the ratio 5:4:1:2:1:2:1. The good fit in this case, contrasting with the poor fit of the F_2 frequencies to the same ratio, is attributed in part to the fact that the F_3 progenies were classified before the effect of the differential in survival among genotypes became pronounced.

TABLE 3.—*Observed distribution of plants of F_3 progenies from 7 F_2 phenotypes from the cross dark red × normal green and χ^2 tests for goodness of fit to ratios expected on the basis of duplicate factors for red*

F_2 phenotype	F_3 progenies	F_3 plants of indicated phenotype							Theoretical ratio	χ^2	P
		Dark red	Intermediate dark red	Red	Intermediate red	Dwarf red	Intermediate dwarf red	Green			
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*			
Dark red	1	7	0	0	0	0	0	0			
	13	183	0	57	0	0	0	0	3:1	0.20	0.50–0.95
	5	40	0	0	0	18	0	0	3:1	1.13	.20–.50
Intermediate dark red	52	460	335	105	186	75	161	79	5:4:1:2:1:2:1	9.72	.10–.20
Red	23	0	0	722	0	0	0	0			
Intermediate red	38	0	0	278	635	0	0	302	1:2:1	3.44	.10–.20
Dwarf red	[1] 6	0	0	0	0	117	0	0			
Intermediate dwarf red	[2] 35	0	0	0	0	254	590	280	1:2:1	3.99	.10–.20
Green	25	0	0	0	0	0	0	735			

[1] 3 additional progenies from plants classed as dwarf red in the F_2 segregated as intermediate dwarf red.
[2] 1 additional progeny from a plant classed as intermediate dwarf red in the F_2 proved to be dwarf red.

Of 19 F_3 progenies from dark-red F_2 plants, 1 did not segregate. The 7 plants in this progeny were all dark-red dwarfs. Thirteen progenies from dark-red F_2 plants segregated for dark red and red, in frequencies showing a good fit to a 3:1 ratio. On closer inspection these 13 progenies were found to be separable into 3 classes—66 dark-red dwarfs, 117 lighter dark-red intermediate dwarfs, and 57 red nondwarfs—frequencies which show a good fit to the ratio 1:2:1 (χ^2=0.82, P=0.50–0.95). The frequencies of the remaining 5 progenies from dark-red F_2 plants showed a good fit to a 3:1 ratio for dark red and dwarf red. On closer study these 5 progenies were found to be separable into 3 classes—12 dark-red dwarfs, 28 lighter dark-red dwarfs, and 18 dwarf reds—frequencies which show a good fit to the ratio 1:2:1 (χ^2=1.31, P=0.50–0.95). These results for the 3 types of progenies from dark-red plants would be expected

from the genotypes postulated in table 1 on the assumption that dwarf stature and red plant color in the dwarf-red phenotype were due to a single factor or to completely linked factors.

According to the theoretical ratios given in table 1 the expected distribution of a total of 19 F_3 progenies among the 3 genotypes $R_1^{RO}R_1^{RO}Rd\ Rd$, $R_1^{RO}R_1^{RO}Rd\ rd$, and $R_1^{RO}r_1^{RO}Rd\ Rd$ would approximate the ratio 4:8:8 if the 3 genotypes were equal in capacity to survive and mature seed. The observed frequencies, 1:13:5, agree with the results for the dwarf-red phenotype in showing lower capacity to reproduce on the part of the genotypes which were homozygous for *Rd*.

Results of the F_3 tests support the tentative finding based on F_2 and backcross tests that the dark-red character is due to the complementary action of two factors affecting anthocyanin pigmentation, one of these, R_1^{RO}, being a factor for red and the other, *Rd*, being a factor for dwarf red.

TESTS FOR INDEPENDENCE OF THE DWARF-RED FACTOR

Absence of linkage between R_1^{RO} and *Rd* is evidenced by the F_3 segregation of the intermediate-dark-red phenotype (table 3), the χ^2 value for which shows a good fit to the ratio expected on the basis of independent inheritance.

Additional tests for independent inheritance of dwarf red were available from the cross of dark red with the linkage tester. Incomplete dominance of L^O and Lc_1^K over their normal allelomorphs and the independent inheritance of these factors, demonstrated by Ware (*9*, *10*), were confirmed by significant χ^2 values for goodness of fit to the monohybrid and dihybrid ratios involved. Dominance of *Fn*, reported by Thadani (*7*) and later workers, and this factor's independence of the three others were similarly confirmed. For convenience in applying the χ^2 tests to determine independence of dwarf red, the factors L^O and Lc_1^K were treated as dominants by including intermediate-okra-leaf plants in the okra classification and including intermediate-brown-lint plants in the brown classification (table 4).

TABLE 4.—χ^2 *tests for the dwarf-red factor's independence of 3 factors of the linkage tester*

[Calculated frequencies based on the observed single-factor segregation in the F_2 from the F_1 hybrid $R_1^{RO}R_1^{RO}\ Rd\ rd\ L^O l^O\ Lc_1^K lc_1^K\ Fn\ fn$]

Segregating factors	Plants of indicated phenotype				χ^2	P
	Observed		Calculated			
	Dark red	Red	Dark red	Red		
Rd and L^O:	*Number*	*Number*	*Number*	*Number*		
Okra [1]	49	40	48.76	40.24	0.01	0.50–0.95
Normal	14	12	14.24	11.76		
Rd and Lc_1^K:						
Brown [2]	46	39	46.57	38.43	.06	.50–.95
Normal	17	13	16.43	13.57		
Rd and *Fn*:						
Naked	41	41	44.92	37.08	2.64	.10–.20
Normal	22	11	18.08	14.92		

[1] Includes intermediate okra. [2] Includes intermediate brown.

Thinning the F_2 population to a single plant per hill altered the ratio of dark-red and red plants (originally 3:1). This invalidated the theoretical 9:3:3:1 dihybrid ratio for testing the dwarf-red factor's independence of the factors of the linkage tester. The observed frequencies were, therefore, tested against frequencies calculated from the observed single-factor segregation of the two pairs of genes in the manner described by Mather (*5*).

Since all F_2 plants were homozygous for R_1^{RO}, the presence of *Rd*, either homozygous or heterozygous, resulted in a dark-red phenotype and its absence resulted in a red phenotype. In table 4, therefore, the dwarf-red factor's independence of the factors of the linkage tester is phenotypically expressed as the independent segregation of dark red. The observed phenotype frequencies do not differ significantly from those expected on the assumption that *Rd* is inherited independently of L^O, Lc_1^K, and *Fn*.

DWARF-RED CHARACTER IN ACALA AND COOK 912

Dwarf red Acala and dwarf red Cook 912 crossed with the linkage tester $R_1^{RO}L^OLc_1^KFn$ gave F_1 progenies which in both cases were darker red than either parent. The F_2 from each cross segregated into seven plant-color classes similar to those of the F_2 from dark red × normal green. Tests for goodness of fit to the 5:4:1:2:1:2:1 ratio expected on the basis of independent inheritance resulted as follows: for the dwarf red Acala cross, $\chi^2=3.22$, $P=0.50$–0.95; for the dwarf red Cook 912 cross, $\chi^2=15.36$, $P=0.01$–0.02.

The backcross to dwarf red Acala of the F_1 from dwarf red Acala × $R_1^{RO}L^OLc_1^KFn$, on the assumption of independent inheritance, would be expected to give dark red, intermediate dark red, dwarf red, and intermediate dwarf red in the ratio 1:1:1:1. The frequencies of a 310-plant population produced by such a backcross showed a good fit to the expected ratio ($\chi^2=5.0$, $P=0.10$–0.20). The frequencies of a 454-plant population produced by a parallel backcross involving dwarf red Cook 912 likewise showed a good fit to the 1:1:1:1 ratio ($\chi^2=1.14$, $P=0.50$–0.95). These results suggest that the mode of inheritance was the same for both dwarf-red phenotypes.

The two backcross populations were classified also as to intermediate-okra versus normal leaf shape. The observed phenotype frequencies, presented in table 5, show a good fit in each case to the 1:1:1:1:1:1:1:1 ratio expected on the basis of independence of the factor for dwarf red and that for okra leaf. These results agree with those for the *Rd* factor of the original dark red.

Dwarf red Acala was distinguishable from dwarf red Cook 912, the latter being slightly darker pigmented and smaller. Mean plant heights of unreplicated plantings near the end of the 1946 growing season were 19.4 inches for dwarf red Cook 912, 28.6 inches for dwarf red Acala, and 22.6 inches for their F_1 hybrid. The F_1 showed no complementary effect of color factors. The F_2 phenotype classes were not clearly defined, owing to the close similarity of the parents. Only 3 classes based on color intensity were distinguished, the Acala parent type, an intermediate, and the Cook 912 parent type. Frequencies of 26, 66, and 40, respectively, showed a good fit ($\chi^2=2.97$, $P=0.20$–0.50) to the ratio 1 : 2 : 1, indicating that the 2 dwarf-red factors were allelomorphic.

TABLE 5.—*Observed plant frequencies for 8 phenotypes from the backcross* r_1^{RO} *Rd* $l^O/R_1^{RO}rdL^O \times r_1^{RO}$ *Rd* l^O/r_1^{RO} *Rd* l^O *and* χ^2 *values for goodness of fit of the joint segregation to the expected 1:1:1:1:1:1:1:1 ratio*

Plant-color and leaf-shape phenotype	Observed frequency of plants from cross involving—	
	Dwarf red Acala	Dwarf red Cook 912
	Number	*Number*
Dark red, normal	45	57
Intermediate dark red, normal	38	57
Dwarf red, normal	43	68
Intermediate dwarf red, normal	48	65
Dark red, intermediate okra	25	58
Intermediate dark red, intermediate okra	30	47
Dwarf red, intermediate okra	36	51
Intermediate dwarf red, intermediate okra	45	51
Total	310	454
χ^2	11.75	6.30
P	0.10–0.20	0.50–0.95

Dwarf red Acala was indistinguishable from the dwarf-red phenotype which segregated out of the original dark red. This fact, together with the evidence from the F_2 and backcross tests, suggests that in these two cases the dwarf-red factors may be identical.

SUMMARY

A dark-red, dwarf phenotype here designated as dark red was isolated at Stoneville, Miss., from a culture of red Acala cotton.

Crossing dark-red with normal green cotton gave seven readily distinguishable color classes in the F_2, whose frequencies suggested that dark red was due to the complementary action of two pairs of factors ($R_1^{RO}R_1^{RO}RdRd$). Observed plant frequencies in a four-color classification of the progeny obtained by backcrossing the F_1 to normal green showed a good fit to the 1 : 1 : 1 : 1 ratio expected from the segregation of two independent pairs of factors.

In the F_2, significant differences in plant height appeared between phenotypes which differed in number of *Rd* (dwarf-red) factors, but not between phenotypes which had the same number of *Rd* factors. The contrast suggests that dwarf stature is closely associated with this factor for red plant color.

F_2 classifications and postulated genotypes were verified by F_3 data, which showed good fit to expected ratios for three F_2 phenotypes and no segregation for three others presumed to be homozygous. One phenotype was shown to consist of three genotypes as postulated.

Results of crossing dark red with the linkage tester $R_1^{RO}L^OLc_1^KFn$ identified the red-plant factor involved in dark red with the factor R_1^{RO} and showed the dwarf-red factor to be independent of the factors for okra leaf, brown lint, and naked seed.

When dwarf red Acala and dwarf red Cook 912, both of which originated as red chimeras on normal green plants, were crossed with the linkage tester bearing the factor R_1^{RO}, they segregated in the F_2 into seven color classes similar to those obtained by crossing dark red with normal green. Results from the backcrosses of the F_1 prog-

enies to the dwarf red parents showed the dwarf-red factor in each case to be independent of the factor for okra leaf.

The frequencies of the F_2 phenotypes from dwarf red Acala × dwarf red Cook 912 showed a good fit to the ratio 1 : 2 : 1, indicating that the dwarf-red factors in these two stocks were allelomorphic.

It is suggested that dwarf red Acala and the dwarf-red phenotype which segregated out of dark red may be controlled by identical factors.

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U. S. GOVERNMENT PRINTING OFFICE: 1949